Before the
DEPARTMENT OF HEALTH AND HUMAN SERVICES
FOOD AND DRUG ADMINISTRATION

ORIGINAL

In re: Food Labeling; Health Claims and Label Statements; Request for Scientific Data and Information	)	**Docket No. 91N-0103**
	)	**(Omega-3 fatty acids and coronary heart disease)**

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SUPPLEMENTAL COMMENTS OF JULIAN M. WHITAKER, M.D.; PURE ENCAPSULATIONS, INC.; WEIDER NUTRITION INTERNATIONAL, INC.; XCEL MEDICAL PHARMACY, LTD; THE AMERICAN PREVENTIVE MEDICAL ASSOCIATION; AND DURK PEARSON AND SANDY SHAW.

Julian M. Whitaker, M.D.; Pure Encapsulations, Inc.; Weider Nutrition International, Inc.; XCEL Medical Pharmacy, Ltd.; the American Preventive Medical Association; and Durk Pearson and Sandy Shaw (collectively the "Joint Commenters"), by counsel and in response to the notice seeking scientific data and information ("Notice") published in the Federal Register, 64 Fed. Reg. 48841-48842 (September 8, 1999) and 65 Fed. Reg. 4252-4253 (January 26, 2000), hereby submit these comments.

I. BACKGROUND OF COMMENTERS

Julian M. Whitaker, M.D. Julian M. Whitaker, M.D. is a physician licensed to practice medicine in the states of California and Washington. He graduated from Dartmouth College in 1966 with a B.S. degree and from Emory University in 1970 with an M.D. degree. He received additional training in surgery as a resident at the University of California Medical School. From 1975 to 1976 he worked as a physician at the

Pritikin Institute in California. Since that time he has been the Clinical Director of the Whitaker Wellness Institute in Newport Beach, California. He is the author of five books: ***Reversing Heart Disease*** (1985), ***Reversing Diabetes*** (1987), ***Reversing Health Risk*** (1989), ***Natural Healing*** (1994), and ***What Your Doctor Won't Tell You About Bypass*** (1995). Since August of 1991 he has been the editor of ***Health & Healing***, currently the nation's largest single editor health newsletter. In 1998, ***Health & Healing*** had over 500,000 subscribers. He receives royalties from the distribution and sale of several dietary supplements based on formulas he develops and licenses. Among the supplements which Dr. Whitaker has formulated (and from which he receives royalty payments) are three containing omega-3 fatty acids. He wants to place the proposed health claim on the labels and in the labeling of his omega-3 fatty acid dietary supplements and, but for FDA's extant bar on labeling use of the claim, would do so. Accordingly, he seeks FDA approval of the claim.

Durk Pearson and Sandy Shaw. Pearson and Shaw are scientists residing in Nevada. They design dietary supplement formulations and license them to manufacturing and retailing companies. They are authors of four books on aging and age-related diseases, including the #1, million plus copy best seller ***Life Extension: A Practical Scientific Approach*** (1982). They have also published three other health books, two of which were best sellers: ***The Life Extension Companion*** (1984); ***The Life Extension Weight Loss Program*** (1986); and ***Freedom of Informed Choice—FDA Versus Nutrient Supplements*** (1993). Durk Pearson and Sandy Shaw were plaintiffs in the *Pearson v. Shalala* case. The agency identifies this proceeding as one to aid it in implementing *Pearson's* mandate. Pearson and Shaw have developed and intend to

license two dietary supplements that contain omega-3 fatty acids. Pearson and Shaw wish to communicate the nutrient/disease relationship that is the subject of these comments on their omega-3 fatty acid dietary supplement labels and in labeling associated with those products.

American Preventive Medical Association. The American Preventive Medical Association (APMA) is a non-profit organization located in Virginia. APMA was founded in October of 1992 and is dedicated to ensuring consumer access to preventive therapies and the rights of health care providers to offer those therapies. APMA was a plaintiff in the Pearson v. Shalala case. The agency identifies this proceeding as one to aid it in implementing *Pearson's* mandate. Several APMA physicians sell dietary supplements that contain omega-3 fatty acids. APMA, and its practitioner members and their hundreds of thousands of patients, would benefit from approval of the health claim that is the subject of this proceeding because it would enable those practitioner members to communicate, and their patients to receive, nonmisleading health information on labels and in labeling concerning the effects of omega-3 fatty acids on reducing the risk of coronary heart disease. APMA and its member physicians, therefore, seek agency approval of the claim.

Pure Encapsulations, Inc. Pure Encapsulations, Inc. (Pure) is a Massachusetts corporation engaged in the business of manufacturing, distributing, and selling over 250 pharmaceutical grade dietary supplements for human and companion animal consumption. Six of the dietary supplements manufactured and sold by Pure contain omega-3 fatty acids. Pure would like to place the proposed health claim that is the subject of this proceeding on the labels and in the labeling of those omega-3 fatty acid products.

Weider Nutrition International, Inc. Weider Nutrition International, Inc. (Weider) is a Utah corporation engaged in the business of manufacturing, distributing, and selling over 2,000 pharmaceutical grade dietary supplements for human and companion animal consumption. Weider has been a health, fitness and sports nutrition leader for nearly fifty years since its founding in 1939. Weider plans to manufacture and sell at least two dietary supplements that contain omega-3 fatty acids. Weider would like to place the proposed health claim that is the subject of this proceeding on the labels and in the labeling of those omega-3 fatty acid products.

XCEL Medical Pharmacy, Ltd. d/b/a XCEL Health Care. XCEL Medical Pharmacy, Ltd. d/b/a XCEL Health Care (XCEL) is a California corporation engaged in the business of manufacturing, distributing, and selling pharmaceutical grade dietary supplements for human consumption. Two of the supplements manufactured and sold by XCEL contain omega-3 fatty acids. XCEL would like to use the health claim that is the subject of this proceeding on the labels and in the labeling of those products.

II. SUMMARY OF THE NOTICE

The Department of Health and Human Services (HHS), Food and Drug Administration (FDA), Center for Food Safety and Applied Nutrition (CFSAN) has published a Notice in the September 8, 1999 Federal Register, 64 Fed. Reg. 48841-48842, requesting scientific data, research study results, and other related information concerning four substance-disease relationships. On January 26, 2000, FDA announced in the Federal Register that it was reopening the comment period and would accept scientific data and written comments that are submitted on or before April 3, 2000. 65 Fed. Reg. 4252. In *Pearson v. Shalala,* 164 F. 3d 650 (D.C. Cir. 1999) *reh'g denied en*

banc, 172 F.3d 72 (D.C. Cir. 1999), the U.S. Court of Appeals for the D.C. Circuit held four FDA sub-regulations (prohibiting each of the four substance-disease relationships) invalid under the First Amendment. See (21 C.F.R. §§ 101.71(a), (c), (e); 101.79 (c)(2)(i)(G)); Pearson, 164 F. 3d 658. One of the four subregulations is the subject of this comment. That regulation, 21 C.F.R. § 101.71(e), prohibits the following claim: "Consumption of omega-3 fatty acids may reduce the risk of coronary heart disease."[1] The FDA Notice states that the agency will determine if an "appropriate scientific basis exists to support the issuance of a proposed rule to authorize a health claim for the relationship between omega-3 fatty acids and heart disease based on the data and information it receives." 64 Fed. Reg. 48841. FDA requests that interested parties submit scientific data and information published between 1992 and the present concerning the relationship.

III. THE PROPER LEGAL ISSUE BEFORE THIS AGENCY IS NOT WHETHER THE CLAIM WILL BE APPROVED BUT, RATHER, WHAT KIND OF DISCLAIMER SHOULD BE USED

Under *Pearson*, this agency must authorize the authorize the Omega-3 fatty acid claim. The Court rejected FDA's argument that the claim was inherently misleading. *Pearson,* 164 F. 3d at 656. The Court determined that the claims were, at worst, potentially misleading. *Pearson,* 164 F. 3d at 656. In accordance with Supreme Court commercial speech precedent, only inherently misleading claims may be suppressed outright. *44 Liquormart v. Rhode Island,* 517 U.S. 484, 503 (1996). Claims that are, at worst, potentially misleading must be authorized with corrective disclaimers. *Pearson,* 164 F. 3d at 657-9. Thus, because the First Amendment – and not the agency's own rules

[1] 21 C.F.R. § 101.71(e) in pertinent part reads: "Health claims not authorized for foods in conventional food form or for dietary supplements of vitamins, minerals, herbs, or other similar substances: Omega-3

and policy preferences – is the Supreme law of the land, this agency must authorize the omega-3 claim. The only legal question confronting the agency is precisely how to disclaim the claim to avoid a misleading connotation. In the first instance, the Court of Appeals has made that decision for the agency. *Pearson,* 164 F. 3d at 658.

IV. FDA MUST IMMEDIATELY AUTHORIZE THE CLAIM ON AN INTERIM BASIS WITH THE DISCLAIMER SPECIFIED BY THE *PEARSON* COURT

The *Pearson* Court held the agency's suppression of the omega-3 claim invalid under the First Amendment to the United States Constitution. *Pearson,* 164 F. 3d at 656-659. It did so upon a complete record including all scientific evidence then before FDA. Having reviewed that evidence and the agency's arguments against claim authorization, it held the claim not inherently misleading but, at worst, only potentially misleading. *Pearson,* 164 F. 3d at 657. Consistent with Supreme Court precedent, a potentially misleading claim must be authorized with disclaimers and may not be suppressed outright. *44 Liquormart v. Rhode Island,* 517 U.S. 484, 503 (1996). Relying on that precedent, the Court of Appeals gave this agency a disclaimer it deemed sufficient to address the agency's concerns about misleadingness. Applied to the omega-3 fatty acid claim, that disclaimer reads: "The evidence is inconclusive because existing studies have been performed with *foods* containing omega-3 fatty acids, and the effect of those foods on reducing the risk of coronary heart disease may result from other components in those foods."

Because the rule FDA now enforces to prevent the claim from appearing on labels and in labeling is invalid, and because the Court has held the claim, at worst, only potentially misleading, FDA must no longer enforce the invalidated rules and must act

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fatty acids and coronary heart disease."

immediately to allow the claim. Prudence dictates, and law necessitates, that this agency allow the claim on an interim basis with the disclaimer the Court crafted to cure potential misleadingness. That will ensure that the First Amendment rights of the Joint Commenters are not violated during the period of agency consideration of alternative disclaimers.

This agency has violated the *Pearson* Court's order by continuing to enforce the invalidated rule on omega-3's from the time of the issuance of the Court's mandate (April 20, 1999) until the present, approximately one year as of the date of these comments. The agency's enforcement of the invalidated rule is an unlawful act that cannot stand. The federal courts have held that violations of constitutional rights, including First Amendment rights, must be rectified with haste and cannot be allowed to stand for years while the Government contemplates its next move. Indeed, the Supreme Court has held that violation of a First Amendment right, even for a very short period of time, constitutes irreparable injury without proof of more. See *Elrod v. Burns,* 427 U.S. 347, 373 (1976) (plurality opinion) ("The loss of First Amendment freedoms, for even minimal periods of time, unquestionably constitutes irreparable injury") quoted in *Jackson v. City of Columbus*, 194 F.3d 737, 747 (6th Cir. 1999); *Iowa Right to Life Comm., Inc. v. Williams,* 187 F.3d 963, 969 (8th Cir. 1999); *Brownsburg Area Patrons Affecting Change v. Baldwin,* 137 F.3d 503, 507 (7th Cir. 1998); *New York Magazine v. Metropolitan Transportation Authority,* 136 F.3d 123, 127 (2nd Cir. 1998); see also *City of Lakewood v. Plain Dealer Publishing Co.,* 486 U.S. 750, 758 (1988); *Washington Free Community v. Wilson,* 426 F.2d 1213, 1218 (D.C. Cir. 1969). When Government violates First Amendment rights, the Supreme Court has held delay in eliminating the rights violation

intolerable: "Speakers . . . cannot be made to wait for years before being able to speak with a measure of security." *Riley v. National Federation of the Blind,* 784 U.S. 781, 793-94 (1988) (internal quotes omitted).

The Supremacy Clause of the Constitution establishes beyond per adventure of doubt that the Constitution and laws in pursuance of it are supreme to contrary laws. U.S. Const. Art. VI, *Marbury v. Madison,* 5 U.S. 137, 178-180 (1803). Accordingly, this agency should not have continued to enforce the invalid rules beyond April 20, 1999, and clearly must immediately authorize the Omega-3 fatty acid claim on an interim basis with the disclaimer specified by the Court of Appeals. At the conclusion of its rulemaking on Omega-3 fatty acids, it may then craft an alternate, final disclaimer, if deemed necessary, to cure any misleadingness the agency perceives based on the supplemental submissions it has solicited.

V. SUPPLEMENTAL SCIENCE CONTINUES TO SUPPORT THE CLAIM

A. RECENT SCIENTIFIC RESEARCH ADDS FURTHER EVIDENCE SUPPORTING HEART DISEASE RISK REDUCTION EFFECTS OF OMEGA-3 FATTY ACIDS

Since the Joint Commenters' initial submission in response to the agency's public notice, additional research has appeared in the peer-reviewed literature supporting the heart disease risk reducing effects of omega-3 fatty acids. Among those studies are the eight described below and appended hereto as Exhibits 2 – 9. Based on the overwhelming body of publicly available scientific evidence, and consistent with the intent of Congress on interpretation of "significant scientific agreement," this agency should reverse its earlier decision and find that significant scientific agreement exists to

support the claim, "Consumption of omega-3 fatty acids may reduce the risk of coronary heart disease." Indeed, so unremarkable is the foregoing claim in the scientific and medical communities today that even the conservative and authoritative *Merck Manual of Diagnosis and Therapy* acknowledges that omega-3 fatty acid supplements lower triglyceride levels and may reduce the risk of coronary heart disease. The *Merck Manual* recommends as accepted medical consensus a diet that includes omega-3 and omega-6 fatty acids for patients at high risk for coronary heart disease (CAD). *See Merck Manual of Diagnosis and Therapy, 17th Edition* (1999) (excerpted and attached hereto as Exhibit 1).

As explained below, even if the agency erroneously fails to approve the claim under its health claims review standard, it must nevertheless authorize it with a reasonable disclaimer because that authorization is required to avoid violation of the First Amendment to the United States Constitution.

In Exhibit 2 hereto (Connor WE, "Importance of n-3 [omega-3] fatty acids in health and disease," *Am J Clin Nutr,* 2000, 71 (1 Suppl.): 171S-175S), a recent review of 38 peer-reviewed scientific articles on the protective effects of omega-3 fatty acids concludes that the "effects of n-3 [omega-3] fatty acids on coronary disease have been shown in hundreds of experiments in animals, humans, tissue culture studies, and even clinical trials." The article supports the conclusion that scientific evidence strongly indicates that omega-3 fatty acids may prevent or ameliorate coronary heart disease and stroke, among other cardiovascular diseases. The author documents overwhelming evidence presented in the reviewed scholarly articles that omega-3 fatty acids act to prevent heart disease through a variety of actions. The author concludes that omega-3

fatty acids: (1) prevent arrhythmias (ventricular fibrillation, which is the most common cause of sudden death, and tachycardia); (2) are precursors to prostaglandins and leukotrienes; (3) have anti-inflammatory properties; (4) inhibit synthesis of cytokines and mitogens; (5) stimulate endothelial-derived nitric oxide; (6) are antithrombotic; (7) have hypolipidemic properties with effects on triacylglycerols and VLDLs; and (8) inhibit atherosclerosis. The author concludes that omega-3 fatty acids help prevent coronary heart disease.

In Exhibit 3 hereto (DeCaterina R, Liao JK and Libby P, "Fatty acid modulation of endothelial activation," *Am J Clin Nutr,* 2000, 71 (1 Suppl.): 213S-223S), the authors describe the results of original research on the ability of fatty acids to retard endothelial activation, the early phase of atherosclerosis. They found that consumption of omega-3 fatty acids reduced endothelial adhesion.

In Exhibit 4 hereto (Goodfellow J, et al., "Dietary Supplementation with Marine Omega-3 Fatty Acids Improve Systemic Large Artery Endothelial Function in Subjects with Hypercholesterolemia," *J Am Coll Cardiol*, 2000, 35: 265-270), the authors describe the results of original research on the effects of omega-3 fatty acid supplementation on the cardiovascular systems of patients with high cholesterol levels. The results of the double-blind placebo controlled study indicate that marine omega-3 fatty acid supplements improve large artery endothelium-dependent dilation in subjects with hypercholesterolemia and do not affect endothelium independent dilation. Dilation and large vessel health are essential for cardiovascular health and the prevention of cardiac events. *Merck Manual, 17th Edition.*

In Exhibit 5 hereto (Kris-Etherton PM, et al., "Polyunsaturated fatty acids in the food chain in the United States," *Am J Clin Nutr,* 2000, 71 (1 Suppl.): 179S-188S), the authors present evidence documenting that the American food supply has experienced a significant decline in the level of omega-3 fatty acids. The "food disappearance data" also indicate that the ratios of omega-6 to omega-3 fatty acids have increased, leading to a less favorable profile for converting alpha-linolenic acid (ALA) to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). To rectify the ratio, fish intake would need to increase four-fold. Supplementation would assist in maintaining favorable ratios and providing the health benefits of omega-3 fatty acids, the authors conclude.

In Exhibit 6 hereto (Nestel PJ, "Fish oil and cardiovascular disease: lipids and arterial function," *Am J Clin Nutr,* 2000, 71 (1 Suppl.): 112S-231S), the author reviews the publicly available peer-reviewed scientific literature concerning the effects of omega-3 fatty acids on lipid profiles and arterial function. The author concludes that omega-3 fatty acids do favorably modify several key risk factors for cardiovascular disease. The author finds that omega-3 fatty acids exhibit a significant antiatherogenic effect by favorably modifying plasma lipids. The author finds that omega-3 fatty acids (1) increase HDL-cholesterol concentrations; (2) reduce plasma triacylglycerol-rich lipoprotein levels; (3) reduce postprandial lipidemia; and (4) reduce lipid remnant concentrations.

In Exhibit 7 hereto (Roche HM and Gibney MJ, "Effect of long-chain n-3 [omega-3] polyunsaturated fatty acids on fasting and postprandial triacyglycerol metabolism," *Am J Clin Nutr*, 2000, 71 (1 Suppl.): 232S-237S), the authors review publicly available peer-reviewed scientific literature concerning the effect of omega-3 fatty acids on plasma triacylglycerol levels. They conclude that the body of evidence

supporting two important postulates ((1) that improved postprandial triacylglycerol metabolism reduces the pathogenesis and progression of coronary heart disease and (2) that non-fasting plasma triacylglycerol levels are a strong and independent predictor of future myocardial infarction) has grown substantially. They find the publicly available evidence supports the conclusion that omega-3 fatty acid supplementation reduces plasma triacylglycerol concentrations.

In Exhibit 8 hereto (Siscovick DS, et al., "Dietary intake of long-chain n-3 [omega-3] polyunsaturated fatty acids and the risk of primary arrest," *Am J Clin Nutr,* 2000, 71 (1 Suppl.) 208S-212S), the authors describe the results of original research on the influence of dietary omega-3 fatty acids from seafood on the incidence of cardiac arrest. The results of the population based case controlled study indicate that omega-3 fatty acids from seafood and supplements derived therefrom are associated with a decreased risk of primary cardiac arrest. The findings suggest to the authors that even comparatively low levels of omega-3 fatty acid intake are linked to a reduced risk of cardiac arrest.

In Exhibit 9 hereto (von Schacky C, "n-3 [omega-3] fatty acids and the prevention of coronary atherosclerosis," *Am J Clin Nutr,* 2000, 71 (1 Suppl.): 224S-227S), the author reviews the publicly available peer-reviewed scientific literature and clinical studies on the effects of omega-3 fatty acids on risk of mortality following a first myocardial infarction. The author concludes that consumption of omega-3 fatty acids may be beneficial in the secondary prevention of coronary artery disease.

B. EVIDENCE OF OMEGA-3'S REDUCTION OF PLATELET AGGREGATION AND ADHESION IS OVERWHELMING AND SUPPORTS THE PROPOSED CLAIM

The scientific evidence presented by the Joint Commenters in response to the agency's notice, in addition to that already before the agency, establish the presence of unequivocal scientific proof that omega-3 fatty acids reduce platelet adhesion and aggregation. (Connor WE, "Importance of n-3 fatty acids in health and disease," *Am J Clin Nutr*, 2000, 71 (1 Suppl.): 171S-175S.) Although FDA appears to accept that omega-3 fatty acids do effect a reduction in platelet adhesion and aggregation it has taken the anomalous, arbitrary and capricious position that reduction in platelet adhesion and aggregation produced by a dietary supplement does not reduce risk of cardiovascular events such as coronary heart disease. The position cannot be maintained in law or logic because the agency has long accepted in the drug context that proof of reduction in platelet adhesion and aggregation justifies approval of therapeutic agents for reduction in the risk of vascular and cardiac events such as stroke and heart attacks. As recently as November 22, 1999, the FDA gave market approval to Aggrenox (a combination of aspirin and diprydamole) to reduce the risk of stroke based on that product's ability to inhibit platelet aggregation and adhesion. On May 18, 1998, FDA gave market approval to Integilin (eptifibatide) to reduce the risk of severe cardiac events based upon proof of eptifibatide's ability to inhibit platelet aggregation. On November 17, 1997, FDA gave market approval to Plavix (clopidogrel bisulfate) to reduce atherosclerotic events such as myocardial infarction, stroke, and vascular death based on the product's platelet aggregation and adhesion inhibitory effects. On March 24, 1993, FDA gave market approval to Ticlid (ticlopidine hcl) for reducing the risk of stroke and cardiovascular

events in high risk patients based on the product's platelet aggregation and adhesion inhibitory effects. FDA's inconsistent position on this mater illustrates the agency's arbitrary and capricious bias against dietary supplements and in favor of prescription drugs.

In approving health claims for soy protein and soluble fiber, FDA stated that those claims were backed by "significant scientific agreement" because the evidence documented their role in reducing LDL cholesterol and increasing HDL cholesterol is linked to a reduction in the risk of cardiovascular disease. 64 Fed. Reg. 57699 (October 26, 1999); 63 Fed. Reg. 8103 (February 18, 1998). Substantial evidence has been presented to the agency that omega-3 fatty acids produce the similar kinds of LDL and VLDL cholesterol reducing and HDL cholesterol increasing effects. . Nestel PJ, "Fish oil and cardiovascular disease: lipids and arterial function," *Am J Clin Nutr*, 2000, 71 (1 Suppl.): 112S-231S. Accordingly, consistent with its prior rulings, FDA should approve the proposed health claim for omega-3 fatty acids. Failure to do so would thus constitute arbitrary and capricious agency action in violation of the Administrative Procedure Act, 5 U.S.C. § 706 (2).

VI. FDA MUST NOT ASSESS "SIGNIFICANT SCIENTIFIC AGREEMENT" BASED ON ITS PROPOSED "GUIDANCE" BECAUSE THE GUIDANCE VIOLATES *PEARSON*, THE INTENT OF CONGRESS, AND THE PLAIN LANGUAGE OF THE NLEA

On December 22, 1999, the FDA published a proposed "Guidance" in a failed attempt to comply with the *Pearson* Court's mandate that it define a standard for "significant scientific agreement." As explained in comments filed by the Joint Commenters in response to that guidance (attached hereto as Exhibit 10 and incorporated herein by reference), FDA may not require near conclusive proof as a condition precedent

to approval of a dietary supplement health claim. Rather, Congress expects this agency to approve claims as backed by "significant scientific agreement" without requiring them to satisfy the standard established by law for FDA approval of drugs (the "substantial evidence" standard in 21 U.S.C. § 355(e)). The bi-partisan Senate Committee on Labor and Human Resources explained in its Committee Report reviewing FDA's application of the health claims standard:

> The committee notes that the significant scientific agreement standard is, by design, more flexible than the standard established by law for FDA to review and approve drugs, which requires a demonstration of safety and effectiveness based on "adequate and well-controlled clinical investigations." While the intake of a nutrient on which a health claim is based must be safe, there is no requirement that health claims be derived from clinical trials, and, by its terms, the standard recognizes that significant scientific agreement on the validity of the claim does not have to be complete. Evidence from a broad range of reliable scientific sources should be considered in determining the adequacy of scientific support.

Senate Report 103-410, at 24.

In its Guidance, the FDA fails to fulfill the *Pearson* Court's order by explaining what "significant scientific agreement" means and what it does not mean. The Guidance does not provide information necessary for regulatees to perceive FDA's guiding principles. While, from the Guidance, the regulated class can understand that FDA views interventional studies involving well designed randomized, controlled clinical trials as its "gold standard," it is entirely impossible from the Guidance to perceive whether FDA will ever accept studies other than interventional or other than those involving randomized, controlled clinical trials, as sufficient for claim authorization. Moreover, FDA requires proof of direct causality (that a substance ***will*** result in a change in a disease endpoint) as a condition precedent to claim approval. A large body of evidence strongly supporting, but not conclusively proving, a substance-disease relationship

appears unlikely to satisfy FDA. Thus, the only principle that regulatees can perceive with clarity from FDA's Guidance is that FDA will accept the same kind of near conclusive proof expected as a condition precedent for drug approval as its basis for dietary supplement claim approval. That principle, however, violates congressional intent as the excerpted passage above makes clear.

Congress plainly expects this agency to authorize health claims for dietary supplements without requiring that those claims be backed by the same kind of near conclusive proof required for the grant of applications for new drugs. Accordingly, to the extent that FDA's Guidance reveals a principle to the regulated class, that principle is one calling for a level of evidence that Congress has unequivocally rejected in the context of health claims for dietary supplements. Consistent with the dictates of Congress, this agency should hold that significant scientific agreement exists when

> a significant segment of scientists having relevant expertise agree, based on relevant scientific evidence, that consumers are *reasonably likely* to obtain the claimed health benefit.

Senate Report 103-410, at 24. Congress has determined that the above-quoted definition which it supplied in committee is "consistent with the NLEA's goal of assuring that consumers have access on food and dietary supplement labels to health claims that are scientifically supported, without having to wait until the degree of scientific certainty contemplated by the drug standard has been achieved." *Id.*

Without question, the overwhelming body of scientific evidence concerning omega-3 fatty acids and coronary heart disease confirms that "a significant segment of scientists having relevant expertise agree, based on relevant scientific evidence, that consumers are *reasonably likely* to obtain the claimed health benefit." Indeed, the

evidence appears to surpass that expected by Congress for claim approval and to approach the near conclusive degree that FDA erroneously expects as a condition precedent for health claim approval. Accordingly, FDA should, indeed it must, approve the claim under 21 U.S.C. § 343(r)(5)(D) and its rules as backed by "significant scientific agreement."

VII. ASSUMING *ARGUENDO* THAT FDA FAILS TO FIND "SIGNIFICANT SCIENTIFIC AGREEMENT," IT MUST NEVERTHELESS AUTHORIZE THE CLAIM WITH DISCLAIMERS CONSISTENT WITH *PEARSON*

Assuming *arguendo* that this agency decides that the Omega-3 claim is not backed by "significant scientific agreement" and, thus, decides not to *approve* it, it may not deny the claim but must nevertheless authorize it with a corrective disclaimer. [CITATION]. Indeed, as explained above, FDA has a constitutional obligation to authorize the claim at the earliest possible moment. In light of the fact that the *Pearson* Court has already determined that the claim is not inherently misleading (CITATION) and is, at worst, only potentially misleading, under applicable First Amendment precedent this agency has an incontrovertible duty to authorize the claim. That duty to authorize the claim trumps any contrary agency preference or rule and necessitates authorization with a disclaimer. U.S. Const. Art. VI, *Marbury* 5 U.S. 178-180 (1803). That duty does not compel FDA to *approve* the claim, as the *Pearson* Court explained. Indeed, if FDA finds "significant scientific agreement" lacking, it may choose not to place its imprimatur of approval upon the claim; nevertheless, even without claim approval under significant scientific agreement, the First Amendment compels FDA to authorize unapproved claims so long as the claims can be rendered nonmisleading through the addition of a disclaimer. *Pearson,* 164 F. 3d at 658-9. In this case, the Court

of Appeals has taken the extraordinary step of fashioning disclaimers for the agency's use. That action, coupled with the First Amendment burden upon government to rectify wrongful acts of suppression with haste, compels FDA to issue immediately an interim rule authorizing the "may" claim with the disclaimer specified by the Court. FDA may then arrest its unlawful enforcement of the constitutionally invalid rule and proceed with rulemaking to define precisely the content of the final disclaimer it desires to require for use with the claim.

VII. CONCLUSION

For the foregoing reasons, FDA must act immediately to authorize the Omega-3 fatty acid "may" claim on an interim basis requiring use of the disclaimer crafted by the *Pearson* Court, as explained above. That action is warranted because the *Pearson* decision invalidated the rule FDA now enforces unlawfully to prevent use of the omega-3 "may" claim. That action is also warranted because First Amendment precedent, cited above, requires immediate elimination of a civil rights violation, including a First Amendment right violation, by this government. Accordingly, FDA should immediately authorize the omega-3 fatty acid claim with the corrective disclaimer specified by the *Pearson* Court. If, upon completion of its rulemaking, it fails to approve the claim under "significant scientific agreement," it must nevertheless authorize it with a disclaimer tailored to satisfy any other reasonable concerns the agency may have. In fact, based on the additional science adduced, FDA should approve the claim without disclaimers in light of the fact that the claim is clearly amply supported by "significant scientific agreement." To avoid a violation of the Administrative Procedure Act's prohibition on arbitrary and capricious agency action, FDA should interpret "significant scientific

of Appeals has taken the extraordinary step of fashioning disclaimers for the agency's use. That action, coupled with the First Amendment burden upon government to rectify wrongful acts of suppression with haste, compels FDA to issue immediately an interim rule authorizing the "may" claim with the disclaimer specified by the Court. FDA may then arrest its unlawful enforcement of the constitutionally invalid rule and proceed with rulemaking to define precisely the content of the final disclaimer it desires to require for use with the claim.

VIII. CONCLUSION

For the foregoing reasons, FDA must act immediately to authorize the Omega-3 fatty acid "may" claim on an interim basis requiring use of the disclaimer crafted by the *Pearson* Court, as explained above. That action is warranted because the *Pearson* decision invalidated the rule FDA now enforces unlawfully to prevent use of the omega-3 "may" claim. That action is also warranted because First Amendment precedent, cited above, requires immediate elimination of a civil rights violation, including a First Amendment right violation, by this government. Accordingly, FDA should immediately authorize the omega-3 fatty acid claim with the corrective disclaimer specified by the *Pearson* Court. If, upon completion of its rulemaking, it fails to approve the claim under "significant scientific agreement," it must nevertheless authorize it with a disclaimer tailored to satisfy any other reasonable concerns the agency may have. In fact, based on the additional science adduced, FDA should approve the claim without disclaimers in light of the fact that the claim is clearly amply supported by "significant scientific agreement." To avoid a violation of the Administrative Procedure Act's prohibition on arbitrary and capricious agency action, FDA should interpret "significant scientific

agreement" as Congress intended. Under the congressionally intended definition, the omega-3 fatty acid claim should be *approved* by the agency. Nevertheless, if it is not *approved,* it should be *authorized* with disclaimers, as required by the First Amendment.

Respectfully submitted,

JULIAN M. WHITAKER, M.D.;
MYCOLOGY RESEARCH LABS LTD;
PURE ENCAPSULATIONS, INC.;
WEIDER NUTRITION INTERNATIONAL, INC.;
XCEL MEDICAL PHARMACY LTD;
THE AMERICAN PREVENTIVE MEDICAL ASSOCIATION; AND
DURK PEARSON AND SANDY SHAW,

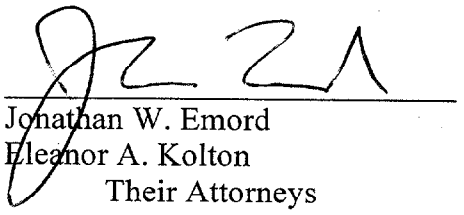

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EXHIBIT 1

Prevention Of Coronary Artery Disease

The Merck Manual of Diagnosis and Therapy
Section 16. Cardiovascular Disorders
Chapter 202. Coronary Artery Disease
Topics
[General]
Prevention Of Coronary Artery Disease
Angina Pectoris
Myocardial Infarction
navigation help

CAD prevention usually begins with reversal of modifiable risk factors. Smoking cessation is of primary importance. Additional strategies include dietary modification, achievement of appropriate weight for height, proper management of stress, and regular exercise. Physicians should treat coexisting disorders associated with increased risk, such as hypertension (see Ch. 199), hypercholesterolemia, diabetes (see Ch. 13), or hypothyroidism (see Ch. 8). In particular, aggressive cholesterol lowering with HMG-CoA reductase inhibitors (statins--see also Ch. 15) has now been demonstrated to save lives, prevent unstable angina and MI, and decrease coronary revascularization rates.

DIETARY MODIFICATION

Fats: The average U.S. diet contains 37% of total calories as fat. The American Heart Association recommends that the proportion be reduced to 30%, yet a reduction to < 10% may be needed to have a major effect on CAD risk.

The type of dietary fat is also important; there are three kinds (Table 202-1): saturated, monounsaturated, and omega-3 and omega-6 PUFAs. The ideal proportion of each of these fats is unknown. However, diets high in saturated fats are clearly atherogenic, and those high in monounsaturates or omega-3 oils are less so.

U.S. studies failed to show a decreased incidence of angina or MI in persons eating diets high in omega-3 oils, although such diets were associated with decreased risk of sudden cardiac death. Persons eating the most fish consumed an average of 0.58 g/day of omega-3 oils, but much higher intakes of omega-3 oils are probably needed for demonstrable risk factor reduction. For example, omega-3 oil supplementation with two or three divided doses of eicosapentaenoic acid 1.8 to 6 g/day and docosahexaenoic acid 0.75 to 2.5 g/day lowers elevated serum triglyceride levels. These doses are up to 10 times the amounts consumed by the fish eaters in the U.S. studies.

For patients at high risk of CAD and especially for those with evidence of CAD, it is reasonable to recommend a 20 g/day fat diet consisting of 6 to 10 g of PUFAs with equal proportions of omega-6 and omega-3 oils, <= 2 g of saturated fat, and the remainder as monounsaturates.

Fruits and vegetables: Five servings/day of fruits and vegetables, which are rich in phytochemicals, seems to decrease the risk of CAD and some cancers. However, populations eating a high phytochemical diet also tend to consume less saturated fat, more fiber, and more vitamin C and E, making the role of phytochemicals less clear. One group of phytochemicals called flavonoids (found in red and purple grapes, red wine, black teas, and dark beers) appear particularly protective against CAD. High intake of flavonoids in red

wine may help explain why French populations have a relatively low incidence of CAD, despite using more tobacco and consuming more fat than Americans do.

Fiber: Americans eat relatively little fiber, of which there are two kinds: soluble fiber (found in oat bran and psyllium), which decreases total cholesterol and may have a beneficial effect on glucose and insulin levels, and insoluble fiber (eg, cellulose, lignin). Fiber is not without adverse effects, however, such as interfering with the absorption of certain minerals and vitamins. In general, foods rich in phytochemicals and vitamins are also rich in fiber.

Vegetable proteins: Consumption of vegetable proteins (eg, soy, tempeh, seitan) seems to decrease CAD risk.

DIETARY SUPPLEMENTATION

Dietary supplementation with vitamins, phytochemicals, omega-3 oils, and trace minerals remains controversial. There are data to justify supplementation with vitamin E, vitamin C, folic acid, and Ca but less convincing data to support the use of vitamin B_6 and B_{12}.

Vitamin E decreases the oxidation of serum LDL-C and thus appears to reduce its capability for vascular damage. Serum vitamin E levels are inversely correlated with incidence of cardiovascular mortality, and supplementation with vitamin E 800 IU/day has been shown to decrease the incidence of MI. A recent study among nurses showed that diets higher in vitamin E were associated with lower death rates from heart disease but failed to show a specific benefit of vitamin E supplementation, possibly because of problems with study design and data collection. Further studies are underway.

Although it has not been shown to decrease the risk of heart disease, supplementation with **vitamin C** 250 to 500 mg bid increases the antioxidant properties of vitamin E.

Folic acid 0.8 mg bid prevents CAD by lowering elevated levels of homocysteine. **Vitamins B_6 and B_{12}** also lower homocysteine levels, but evidence justifying their use in general prevention is scanty. **Calcium** 500 mg bid, aside from its other benefits, appears to have a role in normalizing BP in certain persons.

BIBLIOGRAPHY OF ADDITIONAL REFERENCES FOR OMEGA-3 FATTY ACID/HEART DISEASE HEALTH CLAIM

1. Connor WE, "Importance of n-3 fatty acids in health and disease," *Am J Clin Nutr*, 2000, 71 (1 Suppl.): 171S-175S.
2. DeCaterina R, Liao JK and Libby P, "Fatty acid modulation of endothelial activation," *Am J Clin Nutr*, 2000, 71 (1 Suppl.): 213S-223S.
3. Goodfellow J, et al., "Dietary Supplementation with Marine Omega-3 Fatty Acids Improve Systemic Large Artery Endothelial Function in Subjects with Hypercholesterolemia," *J Am Coll Cardiol,* 2000, 35: 265-270.
4. Kris-Etherton PM, et al., "Polyunsaturated fatty acids in the food chain in the United States," *Am J Clin Nutr*, 2000, 71 (1 Suppl.): 179S-188S.
5. Nestel PJ, "Fish oil and cardiovascular disease: lipids and arterial function," *Am J Clin Nutr*, 2000, 71 (1 Suppl.): 112S-231S.
6. Roche HM and Gibney MJ, "Effect of long-chain n-3 polyunsaturated fatty acids on fasting and postprandial triacylglycerol metabolism," *Am J Clin Nutr*, 2000, 71 (1 Suppl.): 232S-237S.
7. Siscovick DS, et al., "Dietary intake of long-chain n-3 polyunsaturated fatty acids and the risk of primary arrest," *Am J Clin Nutr*, 2000, 71 (1 Suppl.): 208S-212S.
8. von Schacky C, "n-3 fatty acids and the prevention of coronary atherosclerosis," *Am J Clin Nutr*, 2000, 71 (1 Suppl.): 224S-227S.

EXHIBIT 2

Importance of n−3 fatty acids in health and disease[1–3]

William E Connor

ABSTRACT In the past 2 decades, views about dietary n−3 fatty acids have moved from speculation about their functions to solid evidence that they are not only essential nutrients but also may favorably modulate many diseases. Docosahexaenoic acid (22:6n−3), which is a vital component of the phospholipids of cellular membranes, especially in the brain and retina, is necessary for their proper functioning. n−3 Fatty acids favorably affect atherosclerosis, coronary heart disease, inflammatory disease, and perhaps even behavioral disorders. The 38 articles in this supplement document the importance of n−3 fatty acids in both health and disease. *Am J Clin Nutr* 2000;71(suppl):171S–5S.

KEY WORDS n−3 Fatty acids, docosahexaenoic acid, atherosclerosis, coronary heart disease, inflammatory diseases, behavioral disorders

INTRODUCTION

Interest in n−3 fatty acids began some 30 y ago and now culminates in these comprehensive proceedings of the International Conference on Highly Unsaturated Fatty Acids in Nutrition and Disease Prevention, held in Barcelona, November 4–6, 1996. The remarkable concurrence and agreement regarding n−3 fatty acids is evidenced by the several thousand papers extant in the literature.

There is little doubt that n−3 fatty acids are important in human nutrition. They are significant structural components of the phospholipid membranes of tissues throughout the body and are especially rich in the retina, brain, and spermatozoa, in which docosahexaenoic acid (DHA; 22:6 n−3) constitutes ≤36.4% of total fatty acids (1, 2). Membrane fluidity is essential for proper functioning of these tissues. In the retina, where n−3 fatty acids are especially important, deficiency can result in decreased vision and abnormal electroretinogram results. These topics are explored extensively in the supplement.

n−3 Fatty acids are essential fatty acids, necessary from conception through pregnancy and infancy and, undoubtedly, throughout life. It is not known whether there is need in the human diet for the entire spectrum of n−3 fatty acids from the 18-carbon α-linolenic acid (ALA; 18:3n−3) with 3 double bonds to the highly polyunsaturated DHA. Considering that DHA can be synthesized from ALA, is there a need for DHA in infant formulas? Or should DHA be supplied to infant formulas in addition to ALA? DHA is certainly transferred across the placenta to the fetus during pregnancy (3) and is always present in human milk along with other n−3 fatty acids, including ALA. Also, what is the proper ratio in the diet of dietary n−6 to n−3 fatty acids? An imbalance in this ratio can accentuate the n−3 fatty acid deficiency state, as is shown by several review articles in this supplement. The ratio of n−6 to n−3 fatty acids may have increased in industrialized societies because of increased consumption of vegetable oils rich in n−6 fatty acids, ie, linoleic acid (18:2n−6), and reduced consumption of foods rich in n−3 fatty acids. Because both n−3 and n−6 fatty acids are essential, the ratio of arachidonic acid (20:4n−6) to DHA may also be important.

Another important feature of n−3 fatty acids is their role in the prevention and modulation of certain diseases that are common in Western civilization. Evidence of such a role is firm for certain diseases, but only speculative for others. The following is a partial list of diseases that may be prevented or ameliorated with n−3 fatty acids, in descending order of the strength of the available evidence as perceived by this reviewer:

1) coronary heart disease and stroke;
2) essential fatty acid defiency in infancy (retinal and brain development);
3) autoimmune disorders (eg, lupus and nephropathy);
4) Crohn disease;
5) cancers of the breast, colon, and prostate;
6) mild hypertension; and
7) rheumatoid arthritis.

Each of these disease topics and many others are discussed in the various articles in this supplement.

CARDIOVASCULAR EFFECTS OF n−3 FATTY ACIDS

The strongest evidence of a relation between n−3 fatty acids and disease is the inverse relation between the amount of n−3 fatty acids in the diet and in blood and tissues and the occurrence of coronary heart disease and its many complications. Effects of n−3 fatty acids on coronary heart disease have been shown in hundreds of experiments in animals, humans, tissue culture studies, and even clinical trials (4).

Although dietary saturated fat and cholesterol are pathogenic for coronary heart disease, n−3 fatty acids from fish are actually

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protective and, by a variety of mechanisms, prevent deaths from coronary disease, particularly cardiac arrest (5). The unique properties of these fatty acids in coronary heart disease first became apparent in the investigations of the health status of Greenland Eskimos who consumed diets very high in fat from seals, whales, and fish and yet had a low rate of coronary heart disease events (6, 7). Further studies clarified this paradox. The fat the Eskimos consumed contained large quantities of the very-long-chain and highly polyunsaturated fatty acids with 20 and 22 carbons and 5 and 6 double bonds, eicosapentaenoic acid (EPA; 20:5n−3) and DHA, which are abundant in fish, shellfish, and sea mammals and are scarce or absent in land animals and plants. EPA and DHA are synthesized by phytoplankton, which are the plants of the waters and the base of the food chain for marine life. However, the plants of the land also provide a rich source of another n−3 fatty acid, the 18-carbon ALA, from which EPA and DHA may be synthesized and which may itself confer health benefits.

Dietary n−3 fatty acids act to prevent heart disease through a variety of actions (4). They

- prevent arrhythmias (ventricular tachycardia and fibrillation),
- are prostaglandin and leukotrieke precursors,
- have antiinflammatory properties,
- inhibit synthesis of cytokines and mitogens,
- stimulate endothelial-derived nitric oxide,
- are antithrombotic,
- have hypolipidemic properties with effects on triacylglycerols and VLDLs, and
- inhibit atherosclerosis.

EPA and DHA have strong antiarrhythmic action on the heart, as reviewed by Kang and Leaf (5). In experimental animals and tissue culture systems, EPA and DHA prevent the development of ventricular tachycardia and fibrillation. When EPA or DHA is given to isolated, contracting myocytes in culture (induced to ventricular fibrillation by a noxious pharmaceutical agent, ie, ouabain), the fibrillation is aborted.

Even total mortality has been improved in several studies in which the n−3 fatty acid intake was increased. In one study, men who consumed salmon ≥1 time/wk had a 70% less likelihood of cardiac arrest (8). In another study by Burr et al (9), overall mortality was decreased by 29% in men with overt cardiovascular disease who consumed n−3 fatty acids from fish or fish oil, probably because of the reduction in cardiac arrests. In a third study in France, coronary deaths, especially sudden deaths, were prevented by a diet high in ALA (10).

The most recent data on fish consumption and risk of sudden cardiac death were from the Physician's Health Study in the United States in 20551 male physicians (11). Consumption of ≥1 fish meal/wk was associated with a 52% lower risk of sudden cardiac death compared with consumption of <1 fish meal/mo. Total mortality in this sample was also lower in those who ate fish. There did not appear to be a greater reduction in sudden death in those who ate >1 fish meal/wk, suggesting a threshold effect. A similar threshold occurred for intake of n−3 fatty acids. Even a small intake was associated in a reduction in sudden death, from 0.3 to 2.7 g/mo. There was not a reduced risk of total myocardial infarction, nonsudden cardiac death, or total cardiovascular mortality. The limitations of this study were that the nutritional history was taken on entry to the study and then cardiovascular events, including sudden death, were followed up for 11 y. Clearly, fish intake could have varied over this period of time, especially since the study involved physicians, who certainly would have been aware of the overall beneficial effects of fish consumption. There was also no measure of fish-oil supplements, which physicians might have taken in an effort to prevent coronary disease.

Thrombosis is a major complication of coronary atherosclerosis that can lead to myocardial infarction. The n−3 fatty acids from fish oil have powerful antithrombotic actions. EPA inhibits the synthesis of thromboxane A_2 from arachidonic acid in platelets (12). This prostaglandin causes platelet aggregation and vasoconstriction. As a result, fish oil ingestion by humans increases the bleeding time and decreases the stickiness of the platelets for aggregation to glass beads (13). In addition, the administration of fish oil enhances the production of prostacyclin, a prostaglandin that produces vasodilation and less sticky platelets (12). In an in vivo baboon model, dietary fish oil prevented platelet deposition in a plastic vascular shunt (14). Injury to the intima of the carotid artery of the baboon invariably caused a marked proliferative and inflammatory lesion, greatly thickening the wall. When the animals were fed fish oil, such damage and intimal thickening were completely blocked.

The EPA and DHA contained in fish oil fed to experimental animals actually inhibited development of atherosclerosis. There is evidence in both pigs and monkeys that dietary fish oil prevents atherosclerosis by actions other than reducing plasma cholesterol concentrations (15, 16). These actions may be associated with the inhibition of monocyte migration into the plaque, with less cytokine and interleukin 1α production, and through stimulation of the endothelial production of nitric oxide (17). What was previously known as endothelial-derived relaxing factor has been now identified as nitric oxide and the action of this beneficial substance is enhanced by the n−3 fatty acids in fish oil.

Atherosclerotic plaque formation may also be lessened by the reduction in growth factors after fish-oil consumption, particularly platelet-derived growth factor, a potent mitogen for cellular growth (18). Not only is platelet-derived growth factor diminished by fish oil consumption, but its messenger RNA is reduced. Because atherosclerosis begins with cellular proliferation in response to the influx of cholesterol-rich lipoproteins, the inhibition of this proliferation would greatly reduce the growth of the atherosclerotic plaque.

The pronounced effect of fish oil on hyperlipidemia is especially well documented and is supported by results of precise dietary studies in which the effects of a diet rich in salmon oil were compared with those of a vegetable oil and a diet high in saturated fat. Fish oil in particular was shown to lower plasma cholesterol and triacylglycerol concentrations through inhibition of triacylglycerol and VLDL synthesis in the liver (19, 20). Apolipoprotein B production is reduced by consumption of fish oil in comparison with vegetable oils such as safflower or olive oil (21). This mechanism of action is further substantiated by cultures of rabbit and rat hepatocytes in which EPA, in contrast with oleic acid, inhibited triacylglycerol synthesis and stimulated the synthesis of membrane phospholipids (22).

The occasional increase in LDL concentrations that occurs after VLDL and triacylglycerol concentrations are greatly lowered by fish oil is similar to the increase in LDL that occurs after the drug gemfibrozil is given. LDL synthesis and plasma LDL concentrations were reduced after large doses of fish oil were given (23). In contrast with the n−6 fatty acid–rich vegetable oils that lower HDL concentrations, fish oil does not decrease HDL concentrations (19).

Pronounced postprandial lipemia occurs after the fat in high-fat diets is absorbed, and postprandial lipoproteins are known to be atherogenic. They are also thrombogenic because postprandial lipemia increases activated factor VII, a procoagulant (24). Postprandial lipemia from fatty meals of different fats produced similar activation of factor VII (25). Olive oil, touted as a highly beneficial monounsaturated fat, led to just as much activated factor VII as did 4 other fats, including butter. Pretreatment with fish oil greatly lessens postprandial lipemia (26) and this effect should be considered both antiatherogenic and antithrombotic.

As indicated in the report of Hwang et al (27), it is important to ascertain whether n−6 fatty acids from vegetable oils attenuate the beneficial effects of fish and fish oil. Up to 16 g safflower oil, which supplied linoleic acid, was given along with varying amounts of fish oil (6–15 g/d) in this well-controlled metabolic feeding study. The authors documented again some of the beneficial effects of fish oil: reductions in plasma triacylglycerol and plasma fibrinogen even when the diet contained large amounts of linoleic acid–rich safflower oil. However, despite high incorporation of EPA into platelet phospholipids, platelet aggregation and thromboxane B_2 concentrations were unaffected, in contrast with many other studies.

The interactions of dietary saturated fatty acids and fish oil with both thrombotic factors and hyperlipidemia is of interest and was evaluated in healthy men (28). The effects of n−3 fatty acids, principally EPA and DHA, were similar in all diets regardless of variable intakes of saturated fat. The presence of dietary n−3 fatty acids in both the high- and low-saturated-fat diets significantly lowered plasma total cholesterol, VLDL cholesterol, HDL cholesterol, total triacylglycerol, and VLDL triacylglycerol. Because the low-saturated-fat diet decreased total-, LDL-, and HDL-cholesterol concentrations, these results indicated that dietary saturated fats and n−3 fatty acids had independent mechanisms of actions on plasma lipids and lipoproteins. The diet low in saturated fatty acids and high in n−3 fatty acids produced optimal plasma lipid concentrations. The most favorable outcome on platelet function and platelet vascular interactions was obtained when a low-fat diet was supplemented with n−3 fatty acids. A significantly longer bleeding time occurred when n−3 fatty acids were added to a low-saturated-fat diet than when they were added to a diet rich in saturated fats (24). Apparently, a diet high in saturated fats may counteract the beneficial effects of n−3 fatty acids on platelet–vessel wall interactions.

Ideally, the diet best designed to produce the optimal action to prevent cardiovascular disease would be low in saturated fatty acids and high in EPA and DHA from fish or fish oil (28). The low-saturated-fat diet would lower total cholesterol and LDL and the fish oil would lower triacylglycerol and VLDL and have antithrombotic action. However, as already emphasized, the most powerful action of the n−3 fatty acids from fish and fish oil in cardiovascular disease is to prevent ventricular fibrillation and sudden death.

THE ESSENTIALITY OF n−3 FATTY ACIDS AS COMPONENTS OF MEMBRANE PHOSPHOLIPIDS IN INFANCY

There are 2 critical periods for the acquisition of these essential n−3 fatty acids: during fetal development and after birth until the biochemical development in the brain and retina is completed. As already noted, the n−3 fatty acid DHA is an important constituent of the membrane phospholipids of these neural structures, usually occupying the *sn*-2 position. A typical example is phosphatidylethanolamine, which is especially rich in the brain and retina. DHA occupies the 2 position on the glycerol backbone and stearic acid occupies the 1 position of this molecule. Other phospholipids in which DHA is a prominent feature include phosphatidylcholine, or lecithin; phosphatidylinositol; phosphatidylserine; cerebrosides; and sphyingomyelin. There are dozens of different molecular species in the brain and retina, as was denoted in several publications on this subject (29, 30).

n−3 Fatty acid deficiency is manifested in both the blood and in tissue biochemistry (1). Of note is a strikingly low concentration of DHA, which may fall to as much as one-fifth of the normal amount. In addition, the body attempts to replace the deficient DHA with another highly polyunsaturated fatty acid of the n−6 series, docosapentaenoic acid (22:5n−6). Thus, the total polyunsaturated fatty acid content of the membranes may be quite similar, even with a deficiency of DHA, because of its replacement with docosapentaenoic acid. In rhesus monkeys, n−3 fatty acid–deficient diets fed to pregnant animals and then continued after birth induce profound functional changes such as reduced vision, abnormal electroretinograms, impaired visual evoked potential, polydipsia, more stereotypic behavior (eg, pacing), and, perhaps, disturbances of cognition (31, 32). Some of these findings have been replicated in infants fed formulas deficient in n−3 fatty acids (eg, corn- and coconut-oil formulas). However, in human infants the results have been more variable and, obviously, the experimental protocols less rigorous because of ethical considerations. Even so, most studies of premature infants have shown visual impairment and abnormal electroretinograms. In full-term infants the results have been more ambiguous. However, a recent study in full-term infants, in which a standard infant formula was compared with human milk and with formulas enriched with DHA, provided unequivocal evidence of considerable differences in visual evoked potential (33). In all of the human studies, the biochemical evidence in plasma, red blood cells, and, occasionally, in tissues from autopsied infants has substantiated the n−3 fatty acid deficiency state. The lower concentrations of DHA in plasma and erythrocytes are mirrored by lower concentrations in the brain and retina (1). Formula-fed infants have lower concentrations of brain DHA than do infants fed human milk (34, 35). They also have lower intelligence quotients (36).

During pregnancy, both maternal stores and dietary intake of n−3 fatty acids are of importance in insuring that the fetus has adequate amounts of n−3 fatty acids at the time of birth. All the polyunsaturated fatty acids, including DHA, are transferred across the placenta into fetal blood (3). In addition, EPA and DHA in maternal adipose tissue can be mobilized as free fatty acids bound to albumin and be made available to the developing fetus via placenta transport. Several studies in monkeys have indicated that when the maternal diet is deficient in n−3 fatty acids, the infant at birth is likewise deficient as evidenced by low DHA concentrations in their plasma and red blood cells (31). In humans, it was shown that the administration of fish oil or sardines to pregnant women led to higher DHA concentrations in both maternal plasma and red blood cells and in cord blood plasma and red blood cells at the time of birth (37). Once membrane phospholipids have adequate concentrations of DHA, there is an avid retention of these fatty acids in the brain and the retina, even though the diet may subsequently be deficient. Several articles in this supplement illustrate clearly the effects of n−3 deficiency in both animals and humans.

A crucial question for the scientific community and regulatory health bodies throughout the world relates to the amount and kinds of n−3 fatty acids that should be included in infant formulas. It is, of course, completely accepted that infant formulas should contain adequate amounts of n−6 fatty acids. In this context the history of infant formulas is of interest. For many years, infants whose mothers could not feed them human milk were given cow milk diluted with water and reinforced with added sugar (38). These modified cow milk formulas are still used in many parts of the world where poverty or the habits of life prevent the use of commercial infant formulas. The essential fatty acid status of cow milk is questionable, even though the ratio of the n−6 fatty acid linoleic acid to the n−3 fatty acid ALA is appropriate and is in the 2 to 1 range; however, the percentage of energy of essential fatty acids falls far short of World Health Organization recommendations. Modified cow milk has 2% of energy as n−6 and 1% as n−3 fatty acids. Only linoleic acid and ALA are found in cow milk. There are few, if any, detailed studies of the biochemistry and the function of infants fed cow milk.

As noted in the prominent text by Fomon (38) on infant nutrition, infant formulas have undergone many changes since their genesis. A typical corn- and coconut-oil formula has a plethora of linoleic acid and little of ALA. Such formulas were still being marketed up to several years ago in Mexico (32). In the United States, such formulas were changed in the 1980s and soybean oil, which has a good ratio of linoleic acid to ALA (7:1), was introduced. The use of soybean oil has greatly improved the n−3 fatty acid status of currently marketed formulas. The debate now is whether infant formulas could be further improved by the addition of the highly polyunsaturated fatty acids of both the n−3 (DHA) and n−6 (arachidonic acid) categories. This potential improvement in infant formulas, which would make them similar in fatty acid content to human milk, is examined in several articles in this supplement.

CONCLUSION

In summary, n−3 fatty acids have important roles in the modulation and prevention of human diseases, particularly coronary heart disease. Their preventive effects on the brain later in life against disorders such as Alzheimer disease are unknown but are certainly worthy of study. Certainly, the evidence is now strong that n−3 fatty acids are essential for human development in utero and in infancy and are likely to have a role throughout life. The antiarrhythmic effect of n−3 fatty acids is a discovery that has great relevance to the prevention of sudden death from ventricular fibrillation. Further clinical trials in this area are indicated.

REFERENCES

1. Neuringer M, Connor WE, Lin DS, Barstad L, Luck S. Biochemical and functional effects of prenatal and postnatal omega-3 fatty acid deficiency on retina and brain in rhesus monkeys. Proc Natl Acad Sci U S A 1986;83:4021–5.
2. Lin DS, Connor WE, Wolf DP, Neuringer M, Hachey DL. Unique lipids of primate spermatozoa: desmosterol and docosahexaenoic acid. J Lipid Res 1993;34:491–9.
3. Ruyle M, Connor WE, Anderson GJ, Lowensohn RI. Placental transfer of essential fatty acids in humans: venous arterial difference for docosahexaenoic acid in fetal umbilical erythrocytes. Proc Natl Acad Sci U S A 1990;87:7902–6.
4. Connor WE. n−3 Fatty acids and heart disease. In: Kritchevsky D, Carroll KK, eds. Nutrition and disease update: heart disease. Champaign, IL: American Oil Chemists' Society, 1994:7–42.
5. Kang JX, Leaf A. Antiarrhythmic effects of polyunsaturated fatty acids. Circulation 1996;94:1774–80.
6. Bang HO, Dyerberg J. The composition of food consumed by Greenlandic Eskimos. Acta Med Scand 1973;200:69–73.
7. Dyerberg J, Bang HO. Haemostatic function and platelet polyunsaturated fatty acids in Eskimos. Lancet 1979;2:433–5.
8. Siscovick DS, Raghunathan TE, King I, et al. Dietary intake and cell membrane levels of long-chain n−3 polyunsaturated fatty acids and the risk of primary cardiac arrest. JAMA 1995;274:1363–7.
9. Burr ML, Gilbert JF, Holliday RM, et al. Effects of changes in fat, fish, and fiber intakes on death and myocardial reinfarction: Diet and Reinfarction Trial (DART). Lancet 1989;2:756–61.
10. de Lorgeril M, Renaud S, Mamelle N, et al. Mediterranean alpha-linolenic acid–rich diet in secondary prevention of coronary heart disease. Lancet 1994;343:1454–9.
11. Albert CM, Hennekens CH, O'Donnell CJ, Ajani UA, Carey VJ. Fish consumption and risk of sudden cardiac death. JAMA 1998;279:23–7.
12. Goodnight SH Jr, Harris WS, Connor WE, Illingworth DR. Polyunsaturated fatty acids, hyperlipidemia and thrombosis. Arteriosclerosis 1982;2:87–113.
13. Goodnight SH Jr, Harris WS, Connor WE. The effects of dietary omega-3 fatty acids upon platelet composition and function in man: a prospective, controlled study. Blood 1981;58:880–5.
14. Harker LA, Kelly AB, Hanson SR, et al. Interruption of vascular thrombus formation and vascular lesion formation by dietary n−3 fatty acids in fish oil in non human primates. Circulation 1993; 87:1017–29.
15. Weiner BH, Ockene IS, Levine PH, et al. Inhibition of atherosclerosis by cod liver oil in a hyperlipidemic swine model. Circulation 1986;315:841–6.
16. Davis HR, Bridenstine RT, Vesselinovitch D, Wissler RW. Fish oil inhibits development of atherosclerosis in rhesus monkeys. Arteriosclerosis 1987;7:441–9.
17. Shimokawa H, Vanhoutte PM. Dietary omega-3 fatty acids and endothelium-dependent relaxations in porcine coronary arteries. Am J Physiol 1989;256:H968–73.
18. Fox PL, DiCorleto PE. Fish oils inhibit endothelial cell production of platelet-derived growth factor–like protein. Science 1988;241:453–6.
19. Phillipson BE, Rothrock DW, Connor WE, Harris WS, Illingworth DR. Reduction of plasma lipids, lipoproteins, and apoproteins by dietary fish oils in patients with hypertriglyceridemia. N Engl J Med 1985;312:1210–6.
20. Harris WS, Connor WE, Illingworth DR, Rothrock DW, Foster DM. Effect of fish oil on VLDL triglyceride kinetics in man. J Lipid Res 1990;31:1549–58.
21. Nestel PJ, Connor WE, Reardon MR, Connor S, Wong S, Boston R. Suppression by diets rich in fish oil of very low density lipoprotein production in man. J Clin Invest 1984;74:82–9.
22. Benner KG, Sasaki A, Gowen DR, Weaver A, Connor WE. The differential effect of eicosapentaenoic acid and oleic acid on lipid synthesis and VLDL secretion in rabbit hepatocytes. Lipids 1990; 25:534–40.
23. Illingworth DR, Harris WS, Connor WE. Inhibition of low density lipoprotein synthesis by dietary omega-3 fatty acids in humans. Arteriosclerosis 1984;4:270–5.
24. Miller GJ, Martin JC, Mitropoulos KA, et al. Plasma factor VII is activated by postprandial triglyceridemia, irrespective of dietary fat composition. Atherosclerosis 1991;86:163–71.
25. Larsen LF, Bladbjerg EM, Jespersen J, Marckmann P. Effects of dietary fat quality and quantity on postprandial activation of blood coagulation factor VII. Arterioscler Thromb Vasc Biol 1997; 17:2904–9.
26. Harris WS, Connor WE, Alam N, Illingworth DR. The reduction of postprandial triglyceridemia in humans by dietary n−3 fatty acids. J Lipid Res 1988;29:1451–60.

27. Hwang DH, Chanmugam PS, Ryan DH, et al. Does vegetable oil attenuate the beneficial effects of fish oil in reducing risk factors for cardiovascular disease? Am J Clin Nutr 1997;66:89–96.
28. Nordoy A, Hatcher LF, Ullmann DL, Connor WE. Individual effects of dietary saturated fatty acids and fish oil on plasma lipids and lipoproteins in normal men. Am J Clin Nutr 1993;57:634–9.
29. Lin DS, Connor WE, Anderson GJ, Neuringer M. The effects of dietary n−3 fatty acids upon the phospholipid molecular species of monkey brain. J Neurochem 1990;55:1200–7.
30. Lin DS, Anderson GJ, Connor WE, Neuringer M. The effect of dietary n−3 fatty acids upon the phospholipid molecular species of the monkey retina. Invest Ophthalmol Vis Sci 1994;35:794–803.
31. Neuringer M, Connor WE, Van Petten C, Barstad L. Dietary omega-3 fatty acid deficiency and visual loss in infant rhesus monkeys. J Clin Invest 1984;73:272–6.
32. Reisbick S, Neuringer M, Connor WE. Effects of n−3 fatty acid deficiency in nonhuman primates. In: Bindels JG, Goedhardt AC, Visser HKA, eds. Nutricia symposium. Lancaster, United Kingdom: Kluwer Academic Publishers, 1996:157–72.
33. Birch EE, Birch DG, Hoffman DR, et al. Visual maturation of term infants fed n−3 long chain polyunsaturated fatty acid (LCPUFA) supplemented formula. Invest Ophthalmol Vis Sci 1996;37(suppl):S1112 (abstr).
34. Farquharson J, Cockburn F, Patrick WA, Jamieson EC, Logan RW. Infant cerebral cortex phospholipid fatty acid composition and diet. Lancet 1992;340:810–13.
35. Makrides M, Neumann MA, Byard RW, Simmer K, Gibson RA. Fatty acid composition of brain, retina, and erythrocytes in breast- and formula-fed infants. Am J Clin Nutr 1994;60: 189–94.
36. Lucas A, Morley R, Cole TJ, Lister G, Leeson-Payne C. Breast milk and subsequent intelligence quotient in children born preterm. Lancet 1992;339:261–4.
37. Connor WE, Lowensohn R, Hatcher L. Increased docosahexaenoic acid levels in human newborn infants by the administration of sardines and fish oils during pregnancy. Lipids 1996;31(suppl): S183–7.
38. Fomon SJ. Nutrition of normal infants. St Louis: Mosby, 1993.

EXHIBIT 3

Fatty acid modulation of endothelial activation[1–3]

Raffaele De Caterina, James K Liao, and Peter Libby

ABSTRACT Dietary balance of long-chain fatty acids may influence processes involving leukocyte-endothelial interactions, such as atherogenesis and inflammation, that involve increased endothelial expression of leukocyte adhesion molecules, or *endothelial activation*. We compared the ability of various saturated, monounsaturated, and polyunsaturated fatty acids to modulate endothelial activation. Consumption of the n−3 fatty acid docosahexaenoic acid (DHA; 22:6n−3) reduced endothelial expression of vascular cell adhesion molecule 1 (VCAM-1), E-selectin, intercellular adhesion molecule 1 (ICAM-1), interleukin 6 (IL-6), and IL-8 in response to IL-1, IL-4, tumor necrosis factor, or bacterial endotoxin, with a half-maximal inhibitory concentration (IC_{50}) of 1–25 μmol, ie, in the range of nutritionally achievable plasma concentrations. The magnitude of this effect paralleled its incorporation into cellular phospholipids. DHA also reduced the adhesion of human monocytes and monocytic U937 cells to cytokine-stimulated endothelial cells. These effects were accompanied by a reduction in VCAM-1 messenger RNA, indicating a pretranslational effect. To assess structural fatty acid determinants of VCAM-1 inhibitory activity, we compared various saturated, monounsaturated, and n−6 and n−3 polyunsaturated fatty acids for their VCAM-1 inhibitory activity. Saturated fatty acids did not inhibit cytokine-induced expression of adhesion molecules. However, a progressive increase in inhibitory activity was observed with dietary intake of fatty acids with the same chain length but increasing double bonds, ie, from monounsaturated to n−6 and, further, to n−3 fatty acids. Thus, the greater number of double bonds seems critical for the greater activity of n−3 compared with n−6 fatty acids in inhibiting endothelial activation. These properties are likely to be relevant to the antiatherogenic and antiinflammatory properties of n−3 fatty acids. *Am J Clin Nutr* 2000;71(suppl):213S–23S.

KEY WORDS Long-chain fatty acids, atherogenesis, inflammation, endothelium, leukocytes, monocytes, adhesion molecules

INTRODUCTION

Highly unsaturated fatty acids, and n−3 fatty acids in particular, are receiving increasing attention as potential antiatherogenic and antiinflammatory agents. Atherosclerosis and inflammation share similar mechanisms in their early phases, involving increased interactions between vascular endothelia and circulating leukocytes. It was logical, therefore, to investigate a role for fatty acids in the modulation of such interactions. This line of research is leading to a new understanding of the mechanism of action of these nutrients. In this article we will first summarize the biological concepts of the pathogenesis of atherosclerosis. We will then review major findings as to the role of fatty acids in such modulation. Recent findings of ours and of others have led to a new way of thinking about fatty acids and their balance in the diet and, consequently, in membrane phospholipids as modulators of cell responsiveness to cytokines. This concept has broad implications in human pathobiology, nutrition, and therapeutics, with special reference to atherosclerosis and inflammation.

EARLY PHASES OF ATHEROSCLEROSIS

Atherosclerotic lesions originate in discrete points of the arterial tree (mainly branching points, bifurcations, and the convex site of bending arteries) characterized by low or oscillatory shear stresses (1) that can favor the passive transport of arterial blood components into the vessel wall. Late, complex lesions, usually observed in adults, can assume different appearances, reflecting different stages in plaque evolution and perhaps different natural histories in plaque development (2, 3). However, most investigators now agree that arterial fatty streaks represent the earliest stage of plaque development (2, 4–10). This is the earliest detectable lesion in hypercholesterolemic animal models of atherosclerosis in different species (2, 4–10), and is present in the coronary arteries of 50% of young humans between 10 and 14 y of age, as observed in an autopsy study (2). Fatty streaks are areas of focal intimal thickening produced by the intimal accumulation of lipid-laden macrophages (foam cells) surrounded by extracellular matrix and a variable number of lymphocytes. The relation of fatty streaks to more advanced atherosclerotic lesions has long been disputed (11–13) and their full reversibility is generally accepted. However, observations in various animal models (2, 4–10) and particularly in primates (*Macaca nemestrina*) with low-level hypercholesterolemia (8) have clarified that fatty streaks indeed

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precede more advanced atherosclerotic lesions, which develop at the same critical points of the arterial vasculature. Therefore, an understanding of the inception of atherosclerosis requires an understanding of the pathogenesis of fatty streaks (3, 14–17).

Contrary to previous opinions (18, 19), it is now mostly accepted that endothelial damage, in the form of focal desquamation with intimal denudation, is not required and is usually not present at the inception of the atherosclerotic process. Rather, endothelial dysfunction (an earlier, subtler, and more common set of alterations that is not dependent on the physical loss of the endothelial layer) can initiate the entire process. A substantial role is now ascribed, in these early phases, to the monocyte-macrophage (20).

In normal physiologic conditions, the vascular endothelium contributes to vascular homeostasis by adaptively altering its functional state. This happens through a continuous monitoring by the vascular endothelium of blood borne and locally generated stimuli and through an immediate response to changes in its environment (14, 16, 21). Functional properties of the endothelium include active regulation of hemostasis; control of platelet function, coagulation, and fibrinolysis (21); and control of vascular tone, endothelial permeability, and medial smooth muscle cell growth (22). Maladaptive changes in endothelial functions induced by qualitatively or quantitatively abnormal stimuli can result in localized alterations in the interactions of cellular and macromolecular components acting at the blood vessel wall interface, such as changes in the antihemostatic properties of the endothelium, altered control of vascular tone, altered permeability to plasma lipoproteins, hyperadhesiveness to blood leukocytes, and increased cytokine and growth factor production. These alterations can be collectively termed *endothelial dysfunction* (23), a term now used by cardiologists for endothelium-dependent alterations of vascular tone. The term *endothelial activation* more specifically describes the functional changes that endothelia may undergo under the influence of various stimuli—the best studied of which are inflammatory cytokines and bacterial endotoxin—and the acquisition of new functional and antigenic properties, most of which influence interactions with blood leukocytes. Endothelial activation plays an important role in the initiation, progression, and clinical emergence of atherosclerosis (14, 16, 23) and is a pivotal process in monocyte adhesion.

Monocyte binding to the endothelium

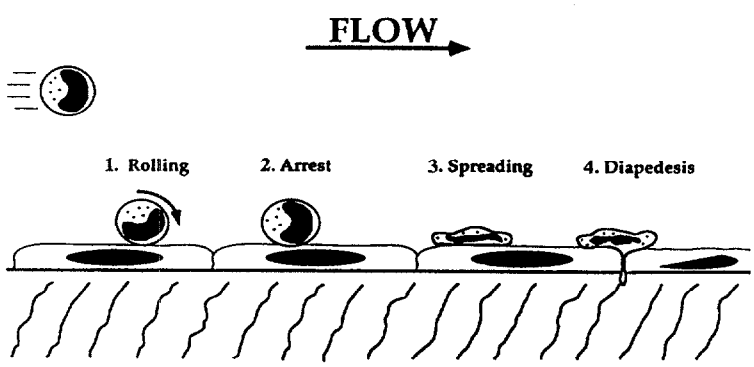
Leukocyte binding to the endothelium is a prominent feature of several inflammatory and immunologic disorders. In acute inflammation, polymorphonuclear leukocytes bind to the endothelium in postcapillary venules. Adhesion of monocytes, but not of polymorphonuclear leukocytes or lymphocytes, to a morphologically normal arterial endothelium is typical of diet-induced atherosclerosis in animals (24). Similarly, many features of the selective recirculation of lymphocytes that occur in a variety of immune reactions are explained by the preferential binding of lymphocyte subtypes to district-specific lymphatic endothelia (25).

Leukocyte binding to cultured endothelial cells has been studied extensively in vitro in an attempt to identify and study the mechanisms mediating this cell-to-cell interaction. It is now clear that activation of leukocytes, endothelial cells, or both can lead to increased adhesion of polymorphonuclear leukocytes, monocytes, or lymphocytes to the endothelium. Several protein families, each with distinct functions, provide "traffic signals" for leukocytes. These include *1*) the selectin family of adhesion molecules, which appear to recognize a sialylated carbohydrate determinant on their cognate ligands (26, 27); *2*) chemoattractants, some of which (classical chemoattractants such as *N*-formyl peptides, complement components, leukotriene B_4, and platelet-activating factor) act broadly on neutrophils, eosinophils, basophils, and monocytes, whereas others [chemokines such as monocyte chemoattractant protein 1 (MCP-1) and interleukin 8 (IL-8)] have selectivity for leukocyte subsets (28, 29); and *3*) the immunoglobulin superfamily members on the endothelium [intercellular adhesion molecule 1(ICAM-1), ICAM-2, ICAM-3, and vascular cell adhesion molecule 1 (VCAM-1)] that recognize integrin ligands on the leukocyte surface in a paradigm first established with ICAM-1 binding to leukocyte function associated antigen 1 (LFA-1) (30; **Figure 1**).

For neutrophil and, probably, lymphocyte adhesion, selectins mediate the initial tethering of the circulating leukocyte over the endothelium, allowing it to roll over the endothelium, slowing down its speed considerably. Antagonists of L-selectin and E-selectin inhibit neutrophil and monocyte influx in response to inflammatory agents (31, 32). Selective targeted disruption of the gene for another such molecule, P-selectin, which is contained preformed in endothelial Weibel-Palade bodies, can also affect leukocyte rolling (33). The slowing down of a leukocyte effected by interactions between selectins and carbohydrates allows the leukocyte to sense the presence of chemotactic gradients and elicit a chemoattractant-receptor–mediated event, ie, the activation of some integrin-type leukocyte ligand exhibiting new activation epitopes (30, 34, 35). Final firm attachment of leukocytes to the endothelium requires the interaction of integrin ligands on the leukocyte surface with immunoglobulin superfamily members, ie, ICAM-1, ICAM-2 and VCAM-1, expressed on the endothelium (30, 36) (**Figures** 1 and **2**).

The possible sequential interactions between selectins and carbohydrates, chemoattractants and receptors, and immunoglobulins and integrins [for neutrophils and possibly also for lymphocyte homing (37, 38)] and the multiple molecular choices available for each of these ligand-to-ligand interactions provide great combinatorial diversity in signals. This diversity allows the selective responses of different leukocyte classes to inflammatory agents, the preferential recirculation patterns of lymphocyte subpopulations, or the selective binding of monocytes to arterial endothelium during early phases of atherogenesis.

Because monocyte recruitment into the intima of large arteries is specific to atherosclerosis but not to other forms of leukocyte-to-endothelium interactions, it was hypothesized that these localized monocyte-to-endothelium interactions reflect specific molecular changes in the adhesive properties of the endothelial surface, leading to expression of "athero-ELAMs," ie, endothelium-leukocyte adhesion molecules (ELAMs) on the endothelial surface in the early phases of atherosclerosis. The first such protein, originally identified in the hypercholesterolemic rabbit model, is VCAM-1 (Figure 2), a member of the immunoglobulin superfamily, expressed on human vascular endothelium in 2 molecular forms (118 and 98 kDa) arising from alternative splicing of unprocessed messenger RNA (mRNA) (39, 40). Both forms are able to bind a heterodimeric integrin receptor, very-late-antigen 4 (VLA_4), with leukocyte selectivity of expression on monocytes and lymphocytes but not on neutrophils. This explains the selective pattern of inhibition of monocyte but not neutrophil adhesion by antibodies directed against VCAM-1 and the selectivity of monocyte recruitment in early atherogenesis (41). Endothelial cells express VCAM-1 early during cholesterol feeding in rabbits, before the



	Rolling	Activation ------>	Firm adhesion	Transendothelial migration
Leukocyte	sLe^x and other sialylated, fucosylated structures L-selectin	Cytokine, chemokine, and chemoattractant receptors	β-1, β-2, and β-3 integrins	PECAM-1 and β-1 and β-2 integrins
Endothelial	P-selectin L-selectin ligand E-selectin CD34 MAdCAM-1	Chemokines (eg, IL-8, MCP-1, MIP-1β) PAF PECAM-1 E-selectin	ICAM-1, ICAM-2, VCAM-1, MAdCAM-1	PECAM-1 ICAM-1 VCAM-1

FIGURE 1. Adhesive interactions during leukocyte (monocyte) emigration. Upper panel: series of events as identified by intravital microscopic studies under flow. The adhesive interactions involved in leukocyte emigration involve several distinct phases: *1*) initial transient adhesion (rolling), *2*) activation, *3*) firm adhesion (arrest and spreading), and *4*) transendothelial migration (diapedesis). Lower panel: molecules involved in these steps. Recent in vitro and in vivo studies indicate that rolling is mediated by multiple low-affinity interactions between selectin receptors and their cognate carbohydrate ligands. Firm adhesion and diapedesis are largely dependent on integrin and immunoglobulin-like proteins. CD34, cluster of differentiation 34; PECAM, platelet–endothelial cell adhesion molecule; ICAM, intercellular adhesion molecule; IL, interleukin; MAdCAM, mucosal adhesion cell adhesion molecule 1; MCP, monocyte chemoattractant protein; MIP, macrophage inhibitory protein; PAF, platelet activity factor; sLe^x, sialyl $Lewis^x$; VCAM, vascular cell adhesion molecule.

appearance of macrophages and foam cells in the intima of a developing fatty streak, in a temporal pattern consistent with its pathogenetic role in lesion development (42).

Interaction between VCAM-1 and VLA_4 is only one of the possible ligand-to-ligand interactions involved in monocyte recruitment in early atherogenesis. The interactions between the regulatable (and, to a large extent, constitutive) endothelial molecule ICAM-1 and the integrin ligands LFA-1 and CD11b/CD18 (Mac-1) (30), between endothelial E-selectin and monocytic sialylated $Lewis^x$ carbohydrate (43), and between monocytic L-selectin and an as yet incompletely characterized inducible endothelial ligand (44) likely contribute to monocyte binding to an activated endothelium. In addition, endothelial monocyte-specific soluble products, which are also inducible by cytokines and endotoxin such as MCP-1 [a monocyte-selective chemoattractant (45)], and macrophage colony-stimulating factor (M-CSF) [able to promote activation and maturation of monocytes and macrophages (46)], are likely to be involved in monocyte recruitment in atherogenesis and have all been detected in atherosclerotic lesions in experimental animals or humans (46–50). So far, VCAM-1 is the only endothelial leukocyte adhesion molecule that appears to be selective for monocytes but not neutrophils.

Endothelial activation as a transducer of atherogenic risk factors

In view of this evidence for the participation of leukocyte adhesion molecules, chemoattractants, and cytokines in early atherogenesis, we must consider the signals that regulate this expression. The gene expression of VCAM-1, as well as that of other adhesion molecules such as ICAM-1, E-selectin, and of inducible soluble endothelial products such as MCP-1, M-CSF, IL-6, and IL-8, is augmented several-fold in response to bacterial endotoxin and cytokines such as IL-1 and tumor necrosis factor (TNF). Resting, unactivated endothelial cells express negligible or low amounts of these molecules, with the notable exception of ICAM-1. After endotoxin and cytokines interact with their specific cell surface receptors, a cascade of intracellular events occurs, ultimately leading to the surface appearance or secretion of these products of endothelial activation. Because most adhesion molecules are not expressed in basal conditions, cytokine activation requires initiation of transcription (51). Also, different adhesion molecules, which are products of separate genes, are expressed simultaneously and in conjunction with increased gene expression of other endothelial products such as MCP-1, M-CSF, IL-6, IL-8, and tissue factor. This leads to the hypothesis that activation of one or several transcription factors, including the early-response genes (c-*jun* and c-*fos*) and the nuclear factor-κB (NF-κB) system, leads to concerted activation of genes. The NF-κB system in particular has received increasing attention over the past several years as a common denominator of endothelial activation and is possibly causally linked with adhesion molecule expression (52).

First discovered in lymphocytes, where it has a role in controlling the activation of genes encoding for the immunoglobulin κ chains (53), the NF-κB system is now known as a much more gen-

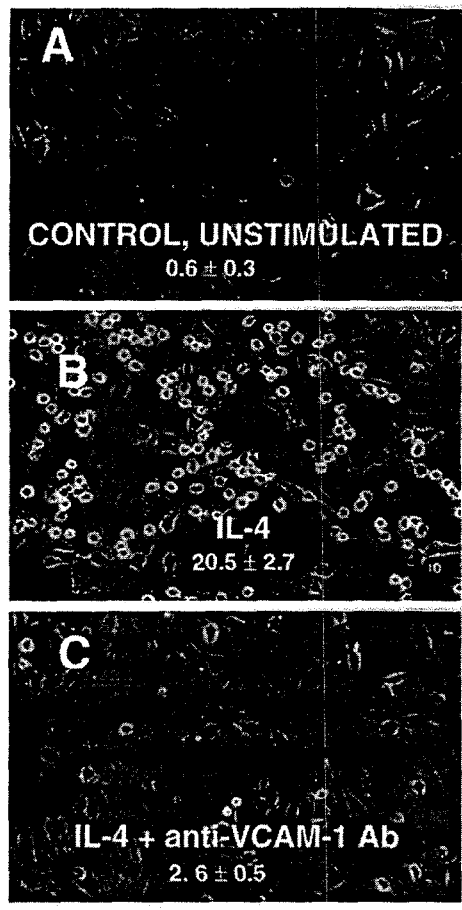


FIGURE 2. The role of vascular cell adhesion molecule (VCAM)-1 in cytokine-induced mononuclear cell adhesion in a typical experiment performed under rotation (63 rpm), mimicking flow conditions somewhat. For these experiments, monocytoid U937 cells were grown in RPMI medium (GIBCO BRL, Grand Island, NY) with 10% fetal calf serum and concentrated by rotation at room temperature and 1×10^9 cells/L. Human saphenous vein endothelial cells were grown to confluence in 6-well tissue culture plates, after which human recombinant interleukin (IL)-4 (Genzyme, Cambridge, MA) was added at 50 μg/L for 24 h. Control endothelial cells do not normally support mononuclear cell adhesion (panel A). Adhesion is dramatically increased after treatment of endothelial cells with cytokines (in this case IL-4; panel B). The increased mononuclear cell adhesion in these conditions is due, to a large extent, to VCAM-1, as assessed by the inhibition obtained in the presence of the anti-VCAM-1 antibody (Ab) E 1/6 (panel C). $\bar{x} \pm$ SEM. In this system, a control nonrelevant antibody (HU.8/4) is completely ineffective (not shown).

eral control mechanism of cell function than originally thought (54). The system consists of a family of transcription factors present in the cytoplasm as dimeric proteins. Normally, these subunits are bound to an inhibiting protein, I-κB. After a phosphorylation of I-κB that allows the attachment of a ubiquitin molecule to the inhibitor and renders it susceptible to proteolytic degradation, the activating subunits are separated from I-κB, allowing the activating subunits to be translocated to the nucleus. There the activating subunits can bind specific "consensus" sequences in the promoter region of several genes that provide a signal necessary for the beginning of mRNA transcription (52). Nucleotide sequences able to bind specifically to NF-κB–like factors (NF-κB elements) have been identified in many human genes, including those for the inducible endothelial leukocyte adhesion molecules and secretable cytokines (52). The NF-κB system provides a potential common link to coordinate the expression of the variety of endothelial genes involved in endothelial activation. Stimuli able to activate the NF-κB system appear also to induce oxidant stress (55, 56) in the form of reactive oxygen species, ie, the superoxide anion and hydrogen peroxide. Antioxidants can inhibit such activation (55, 56), thus giving an important molecular rationale for the therapeutic use of such substances in vascular disease initiation and progression.

A model for other endothelial leukocyte adhesion molecules and cytokine-induced endothelial products, IL-1, TNF, and IL-4 can induce VCAM-1 expression in vitro. These cytokines can be produced by monocyte-macrophages—and, to some extent, by T lymphocytes—infiltrating developing lesions (57). Therefore, such stimuli might provide a paracrine mechanism to amplify the local reaction at the site of a fatty streak, enhancing local monocyte recruitment. The question remains, however, of what initiates the entire atherogenic process. Some hints may come from the notion that cholesterol-induced atherosclerosis in animals is invariably accompanied by both endothelial activation and the focal expression of VCAM-1 (42) and the focal accumulation of LDL in the arterial intima (58). LDL or some of their biotransformation products may stimulate monocyte recruitment. Indeed, several lines of evidence suggest that the critical process that heightens the atherogenicity of LDL is the oxidative modification of LDL in the arterial intima, a microenvironment protected from circulating antioxidants (59–62). Indeed, minimally oxidized LDL or β-VLDL can heighten monocyte adhesiveness to endothelial cells (63), and also increase endothelial production of MCP-1 and M-CSF in vitro (64, 65). As to the exact component of oxidized LDL able to confer such a property, Kume et al (66) reported that a lysophospholipid associated with oxidized LDL particles, lysophosphatidylcholine (alone or in combination with cytokines), can stimulate the expression of some endothelial leukocyte adhesion molecules, including VCAM-1 and ICAM-1, in cultured endothelial cells under certain conditions. Conversely, the protective effect of HDL on atherosclerosis may result in part from inhibition of LDL oxidation (67–69). Other circulating products or metabolites might act by similar mechanisms in conditions associated with enhanced atherosclerotic risk independent of the lipid status. Such factors could include the advanced glycosylation end products associated with diabetes, lipoprotein(a) (a modified LDL particle that appears to be an independent risk factor for atherosclerosis), or homocysteine, as occurs in homocysteinuria and possibly in subtler forms of congenital or acquired enzyme defects in the homocysteine biosynthetic pathway (cystathionine β-synthase or tetrahydrofolate reductase), partly due to vitamin (eg, folate) deficiency. In addition to these humoral stimuli, endothelial gene expression also responds to hemodynamic forces (70, 71), potentially explaining the localization of atherosclerosis at particular points of the arterial vasculature. All these issues are currently under investigation.

Regulation of endothelial activation as a possible mode of action of antiatherogenic substances

Cytokine-induced endothelial activation increases the surface expression of endothelial leukocyte adhesion molecules and the secretion of soluble proatherogenic products (such as MCP-1 and

M-CSF) many-fold. Activated endothelial cells may thus provide a target for therapeutic interventions. In a set of investigations, we showed that several nitric oxide donors can reduce the expression of adhesion molecules and cytokine-inducible, secretable endothelial products by cytokine-activated endothelial (72) and smooth muscle (73) cells. This finding raises the possibility that nitric oxide acts as an endogenous antiatherogenic agent. Subsequent work has shown that these effects occur through induction and stabilization of I-κB, the inhibitor of the transcription factor NF-κB (74). Because NF-κB activation can control the coordinated expression of a variety of adhesion molecules and chemoattractants derived from endothelia or smooth muscle cells, these findings account for a variety of long-term, cyclic guanosine 5′-monophosphate (GMP)-independent actions of nitric oxide in the arterial wall. Sources of nitric oxide in the vasculature include both endothelial cells (mostly by means of the constitutively expressed isoform of the enzyme nitric-oxide synthase now called NOSIII) and other cell types (monocyte-macrophages and smooth muscle cells, mostly by means of the cytokine-inducible nitric oxide synthase called NOSII). The notion of the regulation of endothelial activation by nitric oxide, itself a product of the vessel wall, adds complexity to the entire scheme of the regulation of the expression of ligands and soluble effectors in the origin of fatty streaks. One may speculate that in a normal endothelial cell, endothelium-derived nitric oxide contributes to maintaining an antiatherogenic profile. Conversely, endothelial dysfunction, primarily manifested by an alteration of endothelium-derived vasodilation, also might have a longer-term counterpart in allowing endothelial expression of leukocyte adhesion molecules and chemoattractants. In more general terms, however, the notion of a negative regulation of endothelial activation, as shown by the effects of nitric oxide, identifies a novel, previously unknown mechanism of action for potentially protective factors. Thus, one may speculate that nutritional interventions such as dietary enrichment with L-arginine or administration of novel, long-acting nitric oxide donors may put this novel mechanism to use to counteract the effects of proatherogenic stimuli [such as cytokines, but also, potentially, oxidized LDL, homocysteine, advanced glycation end products of diabetes, and lipoprotein(a)] at the level of the arterial endothelium. By the same token, other known protective factors (ie, estrogens) may act—aside from and beyond influencing plasma lipids—at the level of endothelial activation by influencing leukocyte-endothelial interactions and the start of the response of vessel walls to proatherogenic factors. This view advances our understanding of the initial phases of atherosclerosis, linking biological observations with epidemiologic data and with preventive and, possibly, new therapeutic approaches to this disease.

MODULATION OF ENDOTHELIAL-LEUKOCYTE INTERACTIONS BY n−3 FATTY ACIDS

The epidemiologic evidence of an association between dietary n−3 polyunsaturated fatty acid (PUFA) intake and protection from cardiovascular disease (75–82) is explained, at least in part, by the decreased incidence of atherosclerosis. Apart from numerous animal studies showing decreased atherosclerosis in animals treated with n−3 PUFAs (reviewed in 83), recent evidence has been obtained from human autopsy studies about such effects in Alaskan natives (who consumed high amounts of fish-derived products) and nonnatives (who consume mostly Western-type diets). In the study of Newman et al (84), which reported a decreased percentage of intima covered with fatty streaks and raised lesions in Alaskan natives with high dietary intakes of n−3 PUFAs (80) compared with nonnatives, the magnitude of difference in fatty streak development appeared to be larger in younger age groups (84), suggesting that diet affects mainly the early events leading to fully developed atherosclerotic lesions. A recent study of n−3 PUFA supplementation after coronary bypass surgery indicated that such treatment significantly reduces vein graft stenosis (85), a process that can be regarded as an accelerated form of atherosclerosis.

We therefore hypothesized that n−3 PUFAs may modulate atherogenesis by affecting endothelial activation. We used human adult saphenous vein endothelial cells activated by cytokines in an in vitro model of the early steps in atherogenesis. We first assessed the effects of various fatty acids on the surface expression of endothelial leukocyte adhesion molecules and then characterized the mechanisms and functional relevance of such effects. One n−3 fatty acid, docosahexaenoic acid (DHA; 22:6n−3), when added to cultured endothelial cells hours or days before stimulation with cytokines (early enough to allow a significant incorporation of this fatty acid in cell membrane phospholipids) inhibited events connected with endothelial activation significantly, including the expression of adhesion molecules such as VCAM-1, E-selectin, and, to a lesser extent, ICAM-1, after stimulation with virtually any stimulus able to elicit the coordinated expression of such genes (86, 87). Thus, this inhibition could be shown with IL-1α, IL-1β, TNF, IL-4, and bacterial lipopolysaccharide (**Figure 3**). Inhibition of adhesion molecule expression *1*) occurred in a range of DHA concentrations compatible with nutritional supplementation of this fatty acid to individuals consuming a normal Western diet (Figure 3A), *2*) occurred at any time after the appearance of a cytokine effect, modifying the specific kinetics of surface expression of adhesion molecules (Figures 3, B and C), and *3*) was related in its magnitude strictly to the extent of incorporation into total cell lipids (Figure 3D). Closer analysis of this last relation is shown in **Figure 4**. The extent of the inhibitory effect of VCAM-1 paralleled the incorporation of DHA and the overall increase in incorporation of n−3 PUFAs and was inversely related to the amount of n−6 fatty acids (Figure 4). Experiments assessing the incorporation of [^{14}C]DHA into cell phospholipids showed a significant incorporation of DHA into the phosphatidylethanolamine pool, which is a specific and not particularly abundant phospholipid pool likely to be found in the inner plasma membrane. Therefore, the destination of DHA is possibly a strategic position from which to alter intracellular signal transduction pathways (88; **Figure 5**). This effect was not limited to the expression of transmembrane molecules involved in leukocyte recruitment. The effect was also seen for other cytokine-activated products, ie, the soluble proteins IL-6 and IL-8 (**Figure 6**) involved in either the amplification of the inflammatory response (IL-6; 89) or specific chemoattraction for granulocytes (IL-8; 28), and was accompanied by a functional counterpart, ie, reduced monocyte or monocytoid cell adhesion to cytokine-activated endothelium (**Figure 7**).

One way to unravel the molecular mechanism by which n−3 PUFAs, and DHA in particular, inhibit endothelial activation and VCAM-1 expression is to proceed backward from protein to mRNA analysis and, further, to pathways controlling mRNA accumulation. We first showed that DHA's effects on VCAM-1 expression are accompanied by parallel reductions in VCAM-1 mRNA steady state concentrations, as assessed by Northern

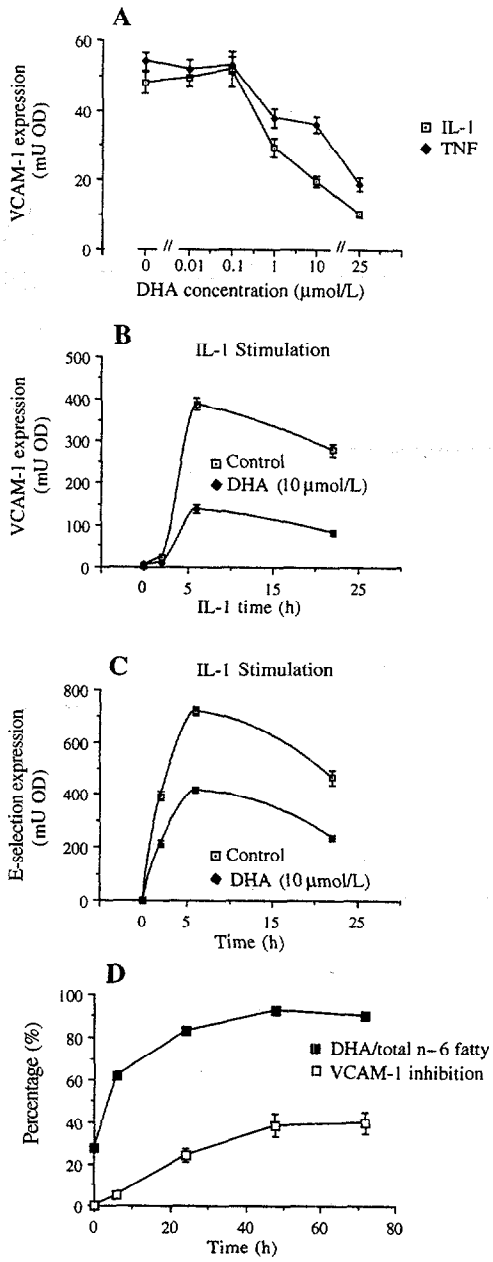


FIGURE 3. Mean (±SEM) inhibition of cytokine-induced expression of VCAM-1 and E-selectin in human saphenous vein endothelial cells by docosahexaenoic acid (DHA). DHA, as sodium salt, was dissolved in medium 199 (Mascia Brunelli, Milan, Italy) containing 5% fetal calf serum and incubated for 96 h with human adult saphenous vein endothelial cell monolayers in 96-well plates, after which cytokines were added for a further 24 h (panel A) or different times (panels B and C) to induce surface expression of endothelial leukocyte adhesion molecules, assessed by cell-surface enzyme immunoassays as described previously (86). A: Dose-response curves of VCAM-1 expression as a function of DHA concentration after stimulation with interleukin (IL)-1α or tumor necrosis factor (TNF)-α, both at 10 μg/L. B and C: Time course of VCAM-1 and E-selectin expression, respectively, after the addition of IL-1α (at 10 μg/L) in the absence or presence of DHA. OD, optical density. D: Parallelism between the inhibition of VCAM-1 expression by DHA and the relative abundance of DHA incorporation in cell membrane lipids, as assessed by gas–liquid chromatographic analysis of total cell-associated lipid extracts. Reproduced, slightly modified, from reference 86 with permission from the American Heart Association.

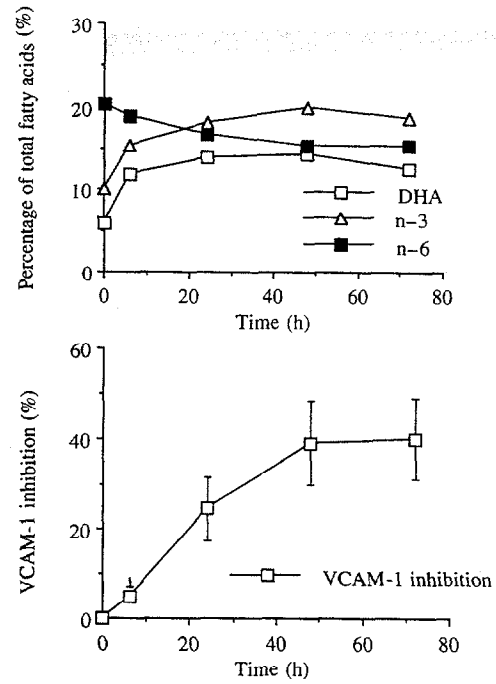


FIGURE 4. Changes in fatty acid incorporation into total cellular lipids as a function of time after incubation of human adult saphenous vein endothelial cells with docosahexaenoic acid (DHA; 10 μmol/L) are shown in the top panel. The time course of the inhibition of surface VCAM-1 expression after stimulation with IL-1 under the same experimental conditions is shown in the bottom panel. Note the parallel increase in DHA and total n−3 fatty acids, and the decrease in total n−6 fatty acids. These changes parallel the progressive increase of VCAM-1 inhibition after IL-1 stimulation.

analysis (86, 87). Similar results from experiments with a remarkably similar design were reported by Weber et al (90). These authors also showed, by using electrophoretic mobility shift assay, an inhibition by DHA of the activation of the NF-κB system of transcription factors (90). These results need to be confirmed. However, potential mechanisms for fatty acid inhibition of the activation of this system of transcription factors on cytokine stimulation can be hypothesized on the basis of comparative experiments that we performed to assess the fatty acid specificity of the effects described.

CONTROL OF ENDOTHELIAL ACTIVATION AS A GENERAL PROPERTY OF UNSATURATED FATTY ACIDS

In earlier experiments, with doses ≤10 μmol/L, DHA appeared to be relatively selective in decreasing cytokine-stimulated VCAM-1 expression [although a synergism with eicosapentaenoic acid (EPA) was already apparent (**Figure 8**)]. To understand whether there was anything specific for DHA in inhibiting cytokine-induced endothelial activation, careful dose-response studies with various fatty acids had to be performed. The availability of VCAM-1 surface enzyme immunoassays, allowing fast processing of 96-well plates of cultured endothelial cells, allowed us to compare the effects of various concentrations of a variety of fatty acids differing in chain length, number, and position of unsaturation. Saturated fatty acids (eg, 16:0, 18:0, and 20:0), monounsaturated fatty acids (eg, *cis*-16:1n−9 and *cis*-18:1n−9), n−6

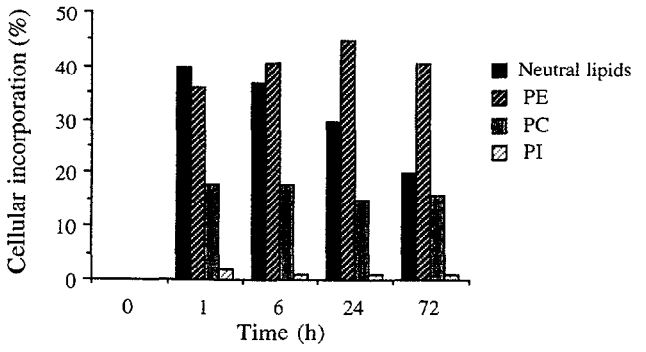


FIGURE 5. Lipid class distribution of [^{14}C]docosahexaenoic acid (DHA) as a function of time in human adult saphenous vein endothelial cells. Major neutral lipids and phospholipid classes [phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI)] were resolved by 2-dimensional thin-layer chromatography, and quantified by counting the radioactivity after scraping the silica plate in areas corresponding to migration of authentic phospholipid standards. DHA accumulated preferentially in neutral lipids at the beginning, then was redistributed into phospholipid classes, with the greatest accumulation in the PE pool.

PUFAs (eg, 18:3n−6 and 20:4n−6), and n−3 PUFAs [eg, 18:4n−3, 20:5 n−3, 22:5n−3 (docosapentaenoic acid; DPA) and DHA] were incubated with human saphenous vein endothelial cells alone for 24–48 h and then in the presence of IL-1 or TNF at 1–10 μg/L for another 24 h. No fatty acids per se elicited endothelial activation as assessed by surface enzyme immunoassay or flow cytometry, nor did saturated fatty acids inhibit cytokine-induced expression of adhesion molecules. However, a progressive increase of inhibitory activity was observed, for the same chain length, with the increase in double bonds accompanying the transition from monounsaturated fatty acids to n−6, and, further, to n−3 PUFAs (**Table 1;** 91). Thus, the greater number of double bonds seems critical for the greater activity of n−3 compared with n−6 fatty acids in inhibiting endothelial activation.

Incidentally, these data imply that such modulatory effects of fatty acids on endothelial activation have little or nothing to do with the generation of eicosanoid mediators, which is another specific property of only some polyunsaturated fatty acids. Indeed, several lines of reasoning argue to exclude a role for eicosanoids in this phenomenon and can be summarized as follows:

1) The effect of DHA is larger than that of EPA. Because EPA is the direct precursor of the 3-series prostaglandins and of the 5-series leukotrienes, one would expect a greater effect of EPA than of DHA if eicosanoids had a role.

2) The effect is unaltered by indomethacin, a blocker of cyclooxygenase (**Figure 9**), which virtually rules out the participation of prostaglandins.

3) The effect is not abolished by eicosatetraenoic acid, a common blocker of all metabolism of arachidonic acid through cyclooxygenase as well as lipoxygenases (data not shown).

4) Although to a lesser extent, the effect was also observed for PUFAs that are not eicosanoid precursors and even in monounsaturated fatty acids, such as oleic acid (86); in this case, a mechanism of action of oleic acid supplementation in the medium would be seen in a relatively selective displacement and substitution of saturated fatty acids in membrane phospholipids, as we reported preliminarily (92).

Thus, the presence of at least one double bond appears to be crucial to these effects of modulation of endothelial-leukocyte interactions. The greater the number of double bonds, the greater the inhibitory effects. n−3 Fatty acids are more active than n−6 fatty acids in this regard because they accomodate more double bonds with the same chain length. One would also predict that substitution of saturated fatty acids in membrane phospholipids, even by monounsaturated fatty acids, would render endothelial cells less responsive to the stimulation of cytokines. These predictions have all been confirmed so far.

POSSIBLE MECHANISMS OF FATTY ACID EFFECTS ON ENDOTHELIAL ACTIVATION

The question now remains, How do fatty acids containing more double bonds in the membrane lipid bilayer lead to diminished activation of the NF-κB system in response to cytokines sufficient to reduce the subsequent start of transcription of genes encoding for endothelial leukocyte adhesion molecules? One possible explanation relates to the intracellular mediators of NF-κB activation,

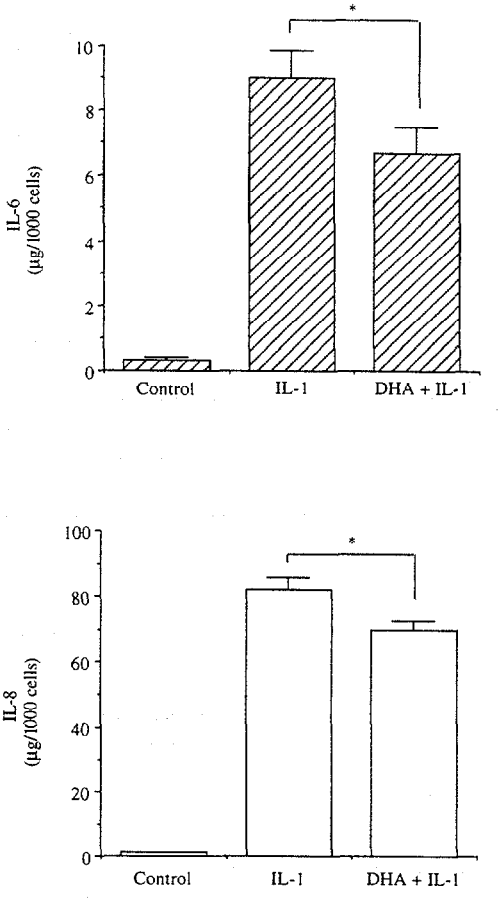


FIGURE 6. Mean (±SEM) production of interleukin (IL)-6 (upper panel) and IL-8 (lower panel) in the absence of endothelial activation (control), after 24-h stimulation with 10 μg IL-1α/L and after docosahexaenoic acid (DHA) (10 μmol/L for 48 h) in conjunction with IL-1α (for a further 24 h still in the presence of DHA). IL-6 and IL-8 were measured in the supernate of endothelial cell cultures. Note the significant decrease in the stimulated production of both IL-6 and IL-8 after preincubation with DHA.

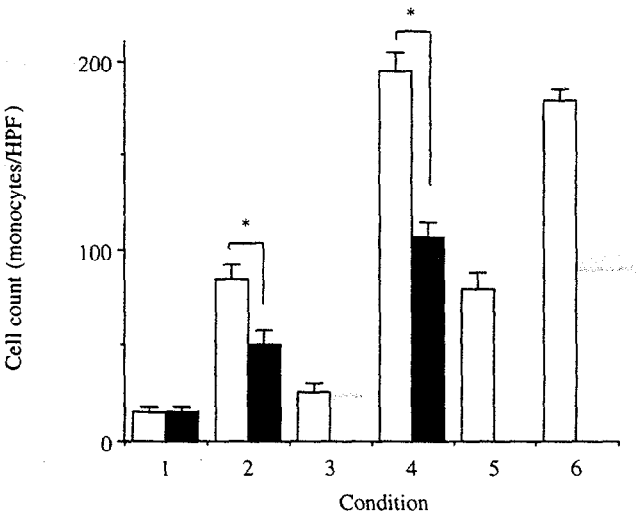


FIGURE 7. Effect of docosahexaenoic acid (DHA) on adhesion of human elutriated monocytes to human adult saphenous vein endothelial cells after stimulation with interleukin (IL)-4 or IL-1, in a typical experiment performed under rotation (63 rpm). Conditions: 1, unstimulated; 2, IL-4 (59 μg/L); 3, IL-4 in conjunction with antibody (Ab) E1/6; 4, IL-1 (10 μg/L); 5, IL-1 in conjunction with Ab E1/6; 6, IL-1 in conjunction with Ab HU8/4. □, Control; ■, DHA; HPF, high-power field; $^*P < 0.05$. Monocyte adhesion in the presence of IL-4 was more dependent on VCAM-1 expression (assessed by the extent of inhibition obtained in the presence of the anti-VCAM-1 antibody E1/6) than in the presence of IL-1. The lack of a response to the control, nonrelevant antibody HU8/4 is also shown. DHA significantly inhibited monocyte adhesion induced by both IL-4 and IL-1. $\bar{x} \pm$ SEM.

namely reactive oxygen species likely formed through the activation of an NADH or NADPH oxidase after cytokine activation. The role of hydrogen peroxide appears to be crucial to this process, whereas its precursor, superoxide anion, appears to have lesser effects, as shown by the almost total abrogation of cell activation of cytokines by cell-permeable catalase (polyethylene glycol-conjugated catalase) and, conversely, the lack of action of polyethylene glycol-superoxide dismutase (72). The marginal role of the superoxide dismutase–mediated scavenging effect of O_2- could be accounted for by the spontaneous alternative dismutation of O_2- likely to occur at acidic intracellular pH. A scavenging effect of O_2- in this system in the presence of the nitric oxide radical is likely to account for the inhibition of NF-κB activation, possibly through enhanced transcription or stabilization of the inhibitor I-κB (74). It is conceivable that similar oxygen-scavenging reactions occur with unsaturated fatty acids. These would lead on the one hand to the initiation of fatty acid peroxidation and on the other hand the prevention of O_2- from generating hydrogen peroxide and by this

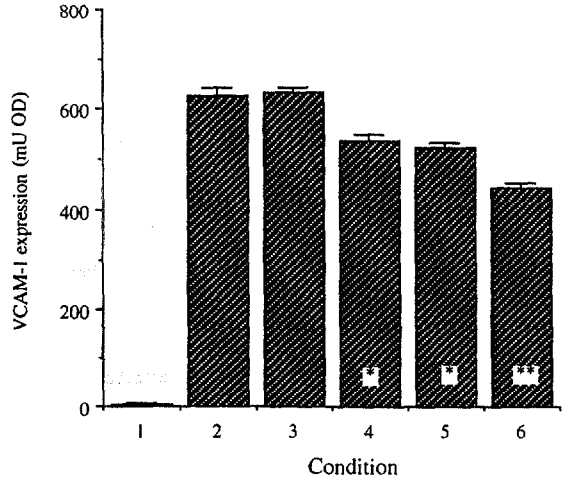


FIGURE 8. Mean (±SEM) effect of eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids in combination compared with either agent alone on interleukin-1 (IL-1)–stimulated vascular cell adhesion molecule 1 (VCAM-1) expression. The experimental design was similar to that described in Figure 3A, with a preincubation time of 24 h before the addition of IL-1. Conditions: 1, control, unstimulated ($n = 8$); 2, control plus IL-1 ($n = 10$); 3, EPA (10 μmol/L) plus IL-1 ($n = 20$); 4, DHA (10 μmol/L) plus IL-1 ($n = 20$); 5, EPA plus DHA (both 5 μmol/L) and IL-1 ($n = 29$); 6, EPA plus DHA (both 10 μmol/L) and IL-1 ($n = 30$). Although EPA was ineffective at 10 μmol/L, a synergism between the 2 fatty acids was apparent when they were used in combination. *,**Significantly different from the control plus IL-1 group, $^*P < 0.05$, $^{**}P < 0.01$ (Sheffe's test after ANOVA).

TABLE 1
Effects of n−6 and n−3 fatty acids, compared with saturated fatty acids, on vascular cell adhesion molecule 1 (VCAM-1) expression by human saphenous vein endothelial cells after incubation with 1 μg interleukin 1α

Fatty acid (25 μmol/L)	VCAM-1 inhibition
	%
Arachidonic (20:4n−6)	15 ± 4[a]
Eicosapentaenoic (20:5n−3)	25 ± 9[b]
Docosapentaenoic (22:5n−3)	28 ± 12[b]
Docosahexaenoic (22:6n−3)	48 ± 18[c]

[1] $\bar{x}$ ± SEM; $n = 12$. $P < 0.01$ for each condition, one-way ANOVA. Differences between arachidonic acid and the others (a), between eicosapentaenoic and docosahexaenoic acids (b), and between docosapentaenoic and docosahexaenoic acids (c) are significant, $P < 0.05$ (Student's t test after Bonferroni's correction). Data from reference 91.

mechanism prevent cell activation. Alternatively, it might be that PUFAs induce some hydrogen peroxide–degrading enzyme, eg, glutathione peroxidase (93). These hypotheses are currently being tested.

A BROADER PERSPECTIVE ON FATTY ACIDS AS MODULATORS OF CELL ACTIVATION

We can now formulate the broader concept that fatty acids may act as modulators of cell responsiveness to cytokines. This concept is entirely original and attractive because it can coherently explain several previously unconnected observations, specifically, *1*) the reduced production of IL-1 and TNF by monocytes stimulated with bacterial lipopolysaccharides (94), *2*) the reduced expression of tissue factor activity by monocytes (95), *3*) the reduced accumulation of platelet-derived growth factor (PDGF) mRNA in mononuclear cells (96), and *4*) the reduced in vivo adhesion of leukocytes in hamsters (97). Our theory also allows us to predict that other cytokine-induced products of endothelial cells, leukocytes, and other cytokine-responsive cells (eg, fibroblasts and smooth muscle cells) could be affected by similar mechanisms. Actually, by one of these mechanisms, fatty acids may control eicosanoid production by a mechanism different from and unrelated to substrate availability. The recent notion that cytokines such as IL-1 and phorbol esters increase the capacity of endothelial cells (and possibly other cell types) to produce prostaglandins via the induction of a recently discovered second cyclooxygenase enzyme [prostaglandin G/H synthase II, also termed cyclooxygenase 2 (98–100)], leads us to hypothesize that such synthesis may also be inhibited by DHA. The finding that DHA, but not EPA, is able to decrease endothelial surface expression of adhesion molecules could also be reverified with regard to prostaglandin G/H synthase II. If so, it might well lead to other research directions. EPA and DHA have always been referred together as n−3 PUFAs, implying similar spectra of biological and pharmacologic profiles. None of the available dietary supplements with n−3 PUFAs presently use the notion of a biologically important difference in the action of these 2 compounds. Research on ways to exploit the peculiar properties of individual fatty acids would therefore be warranted. For a complete structure-relation analysis of the inhibitory properties on endothelial activation of fatty acids, *see* reference 101.

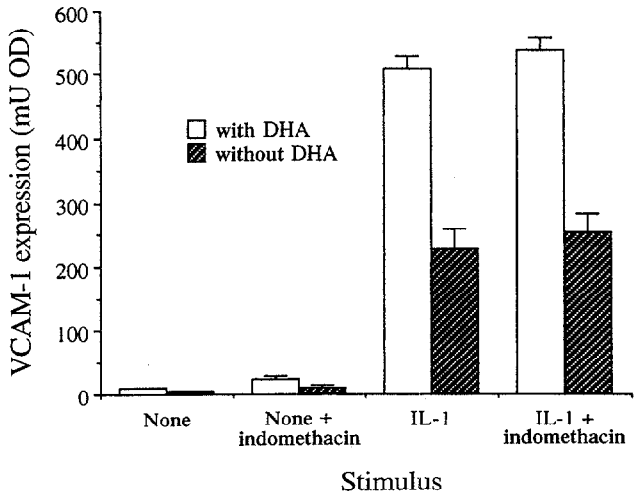


FIGURE 9. Mean (±SEM) inhibition of vascular cell adhesion molecule 1 (VCAM-1) expression by docosahexaenoic acid (DHA) in the absence or presence of 5 μmol indomethacin/L. Note that DHA inhibition of VCAM-1 expression was unaffected by indomethacin.

REFERENCES

1. Ku DN, Giddens DP, Zarins CK, Glagov S. Pulsatile flow and atherosclerosis in the human carotid bifurcation: positive correlation between plaque location and low and oscillating shear stress. Arteriosclerosis 1985;5:293–302.
2. Stary H. Evolution and progression of atherosclerotic lesions in coronary arteries of children and young adults. Arteriosclerosis 1989;88(suppl):1–32.
3. Fuster V, Badimon L, Badimon JJ, Chesebro JH. The pathogenesis of coronary artery disease and the acute coronary syndromes (first of two parts). N Engl J Med 1992;326:242–51.
4. Gerrity RG. The role of the monocyte in atherogenesis: I. Transition of blood-borne monocytes into foam cells in fatty lesions. Am J Pathol 1981;103:181–90.
5. Joris I, Znad T, Nunnari JJ, Krolikowski FJ, Majno G. Adhesion and emigration of mononuclear cells in the aorta of hypercholesterolemic rats. Am J Pathol 1983;113:341–58.
6. Faggiotto A, Ross R, Harker L. Studies of hypercholesterolemia in the nonhuman primate. I. Changes that lead to fatty streak formation. Arteriosclerosis 1984;4:323–40.
7. Rosenfeld ME, Tsukada T, Gown AM, Ross R. Fatty streak initiation in Watanabe heritable hyperlipemic and comparably hypercholesterolemic fat-fed rabbits. Arteriosclerosis 1987;7:9–23.
8. Masuda J, Ross R. Atherogenesis during low level hypercholesterolemia in the nonhuman primate. I. Fatty streak formation. Arteriosclerosis 1990;10:164–77.
9. Faggiotto A, Ross R. Studies of hypercholesterolemia in the nonhuman primate. II. Fatty streak conversion to fibrous plaque. Arteriosclerosis 1984;4:341–56.
10. Masuda J, Ross R. Atherogenesis during low level hypercholesterolemia in the nonhuman primate. II. Fatty streak conversion to fibrous plaque. Arteriosclerosis 1990;10:178–87.
11. Mitchell JRA, Schwartz CJ. Arterial Disease. Oxford, UK: Blackwell Scientific Publishers, 1965.
12. Smith EB, Slater RS. Lipids and low-density lipoproteins in intima in relationship to its morphological characteristics. In: Schletter D, Weizel A, eds. Atherogenesis: initiating factors. Ciba Foundation symposium no. 12. Amsterdam: Elsevier Publishing Co, 1973:39–51.
13. Smith EB, Slater RS, Crothers DX. Quantitative interrelationships between plasma constituents and normal and atherosclerotic human intimal tissue. In: Schletter D, Weizel A, eds. Atherosclerosis III. Berlin: Springer-Verlag, 1974:96–102.

14. Gimbrone MA Jr. Endothelial dysfunction and the pathogenesis of atherosclerosis. In: Fidge N, Nestel P, eds. Atherosclerosis VII. New York: Elsevier Science Publishers, 1986:367–9.
15. Ross R. The pathogenesis of atherosclerosis: an update. N Engl J Med 1986;314:488–500.
16. Gimbrone MA Jr. Endothelial dysfunction and atherosclerosis. J Cardiac Surg 1989;4:180–3.
17. De Caterina R, Gimbrone MA Jr. Leukocyte-endothelial interactions and the pathogenesis of atherosclerosis. In: Kristensen SD, Schmidt EB, De Caterina R, Endres S, eds. n−3 Fatty acids—prevention and treatment in vascular disease. London: Springer Verlag, 1995:9–24.
18. Ross R, Glomset J. The pathogenesis of atherosclerosis. Part I. N Engl J Med 1976;295:369–77.
19. Ross R, Glomset J. The pathogenesis of atherosclerosis. Part II. N Engl J Med 1976;295:420–5.
20. Libby P. Atheroma: more than mush. Lancet 1996;248(suppl):S4–7.
21. Gimbrone MA Jr, ed. Vascular endothelium in hemostasis and thrombosis. Edinburgh: Churchill Livingstone, 1986.
22. Gimbrone MA Jr, Bevilacqua MP. Vascular endothelium: functional modulation at the blood interface. In: Simionescu N, Simionescu M, eds. Endothelial cell biology in health and disease. New York: Plenum Press, 1988:255–73.
23. Gimbrone MA Jr, Kume N, Cybulsky MI. Vascular endothelial dysfunction and the pathogenesis of atherosclerosis. In: Weber P, Leaf A, eds. Atherosclerosis reviews. Vol 25. New York: Raven Press, 1993.
24. Cybulsky MI, Gimbrone MA Jr. Endothelial-leukocyte adhesion molecules in acute inflammation and atherogenesis. In: Simionescu N, Simionescu M, eds. Endothelial cell dysfunctions. New York: Plenum Press, 1992:129–40.
25. Mackay C. Migration pathways and immunologic memory among T lymphocytes. Semin Immunol 1992;4:51–8.
26. Lasky L. Selectins: interpreters of cell-specific carbohydrate information during inflammation. Science 1992;258:964–9.
27. Rosen S. Cell surface lectins in the immune system. Semin Immunol 1993;5:237–47.
28. Oppenheim JJ, Zachariae CO, Mukaida N, Matsushima K. Properties of the novel proinflammatory supergene "intercrine" cytokine family. Annu Rev Immunol 1991;9:617–48.
29. Miller M, Krangel M. Biology and biochemistry of the chemokines: a family of chemotactic and inflammatory cytokines. CRC Crit Rev Immunol 1992;12:17–46.
30. Springer TA. Adhesion receptors of the immune system. Nature 1990;346:425–34.
31. Mulligan M, Varani J, Dame M, et al. Role of endothelial-leukocyte adhesion molecule 1 (ELAM-1) in neutrophil-mediated lung injury in rats. J Clin Invest 1991;88:1396–406.
32. Picker L, Butcher E. Physiological and molecular mechanisms of lymphocyte homing. Annu Rev Immunol 1992;10:561–91.
33. Mayadas T, Johnson R, Rayburn H, Hynes R, Wagner D. Leukocyte rolling and extravasation are severely compromised in P selectin-deficient mice. Cell 1993;74:541–54.
34. Landis R, Bennett R, Hogg N. A novel LFA-1 activation epitope maps to the I domain. J Cell Biol 1993;120:1519–27.
35. Diamond MS, Springer TA. A subpopulation of Mac-1 (CD11b/CD18) molecules mediates neutrophil adhesion to ICAM-1 and fibrinogen. J Cell Biol 1993;120:545–56.
36. Chan BMC, Elices MJ, Murphy E, Hemler ME. Adhesion to vascular cell adhesion molecule 1 and fibronectin: comparison of alpha 4 beta 1 (VLA-4) and alpha 4 beta 7 on the human B cell line JY. J Biol Chem 1992;267:8366–70.
37. Springer TA. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell 1994;76:301–14.
38. Luscinskas FW, Gimbrone MA Jr. Endothelial-dependent mechanisms in chronic inflammatory leukocyte recruitment. Annu Rev Med 1996;47:413–21.
39. Cybulsky MI, Fries JWU, Williams AJ, et al. Alternative splicing of human VCAM-1 in activated vascular endothelium. Am J Pathol 1991;138:815–23.
40. Cybulsky MI, Fries JWU, Williams AJ, Sultan P, Gimbrone MA Jr, Collins T. Gene structure, chromosomal location, and basis for alternative mRNA splicing of the human VCAM-1 gene. Proc Natl Acad Sci U S A 1991;88:7859–63.
41. Bochner BS, Luscinskas FW, Gimbrone MA Jr, et al. Adhesion of human basophils, eosinophils, and neutrophils to interleukin-1-activated human vascular endothelial cells: contribution of endothelial cell adhesion molecules. J Exp Med 1991;173:1553–6.
42. Li H, Cybulsky MI, Gimbrone MA Jr, Libby P. An atherogenic diet rapidly induces VCAM-1, a cytokine-regulatable mononuclear leukocyte adhesion molecule, in rabbit aortic endothelium. Arterioscler Thromb 1993;13:197–204.
43. Erbe DV, Wolitzky BA, Presta LG, et al. Identification of an E-selectin region critical for carbohydrate recognition and cell adhesion. J Cell Biol 1992;119:215–27.
44. Spertini O, Luscinskas FW, Kansas GS, et al. Leukocyte adhesion molecule-1 (LAM-1, L-selectin) interacts with an inducible endothelial cell ligand to support leukocyte adhesion. J Immunol 1991; 147: 2565–73.
45. Rollins BJ. JE/MCP-1: an early response gene encodes a monocyte-specific cytokine. Cancer Cells 1991;3:517–24.
46. Clinton SK, Underwood R, Sherman ML, Kufe DK, Libby P. Macrophage-colony stimulating factor gene expression in vascular cells and in experimental and human atherosclerosis. Am J Pathol 1992;140:301–16.
47. Cybulsky MI, Gimbrone MA Jr. Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. Science 1991;251:788–91.
48. Rosenfeld M, Ylä-Herttuala S, Lipton B, Ord V, Witztum J, Steinberg D. Macrophage colony-stimulating factor mRNA and protein in atherosclerotic lesions of rabbits and humans. Am J Pathol 1992; 140:291–300.
49. Poston R, Haskard D, Coucher J, Gall N, Johnson-Tiedy R. Expression of intercellular adhesion molecule-1 in atherosclerotic plaques. Am J Pathol 1992;140:665–73.
50. Nelken N, Coughlin S, Gordon D, Wilcox J. Monocyte chemoattractant protein-1 in human atheromatous plaques. J Clin Invest 1991;88:1121–7.
51. Kishimoto T, Taga T, Akira S. Cytokine signal transduction. Cell 1994;76:253–62.
52. Collins T. Endothelial nuclear factor-kappa B and the initiation of the atherosclerotic lesion. Lab Invest 1993;68:499–508.
53. Bauerle PA. The inducible transcription activator NF-kappa B: regulation by distinct protein subunits. Biochim Biophys Acta 1991; 1072:63–80.
54. Lenardo M, Baltimore D. NF-kappa B: a pleiotropic mediator of inducible and tissue specific gene control. Cell 1988;58:227–9.
55. Marui N, Offermann MK, Swerlick R, et al. Vascular cell adhesion molecule-1 (VCAM-1) gene transcription and expression are regulated through an antioxidant-sensitive mechanism in human vascular endothelial cells. J Clin Invest 1993;92:1866–74.
56. Collins T, Read MA, Neish AS, Whitley MZ, Thanos D, Maniatis T. Transcriptional regulation of endothelial cell adhesion molecules: NF-kappa B and cytokine-inducible enhancers. FASEB J 1995;9:899–909.
57. Clinton SK, Libby P. Cytokines and growth factors in atherogenesis. Arch Pathol Lab Med 1992;116:1292–300.
58. Schwenke D, Carew TE. Initiation of atherosclerotic lesions in cholesterol-fed rabbits. II. Selective retention of LDL vs. selective increases in LDL permeability in susceptible sites of arteries. Arteriosclerosis 1989;9:908–18.
59. Witztum J. The role of monocytes and oxidized LDL in atherosclerosis. In: Leaf A, Weber P, ed. Atherosclerosis reviews: prevention and noninvasive therapy of atherosclerosis. Vol 21. New York: Raven Press, 1990:59–69.
60. Witztum JL, Steinberg D. Role of oxidized low-density lipoprotein in atherogenesis. J Clin Invest 1991;88:1785–92.
61. Witztum JL. The oxidation hypothesis of atherosclerosis. Lancet 1994;344:793–5.
62. Parthasarathy S. Mechanism(s) of cell mediated oxidation of low density lipoprotein. In: Nohl H, Esterbauer H, Evans CR, eds. Free

radicals in the environment, medicine and toxicology. London: Richelieu Press, 1994:163–79.
63. Territo M, Berliner J, Almada L, Ramirez R, Fogelman A. Beta-VLDL pretreatment of endothelial monolayers increases monocyte but not neutrophil adhesion. Arteriosclerosis 1989;9:824–8.
64. Cushing SD, Berliner JA, Valente AJ, et al. Minimally modified LDL induces monocyte chemotactic protein 1 in human endothelial and smooth muscle cells. Proc Natl Acad Sci U S A 1990;87:5134–8.
65. Rajavashisth TB, Andalibi A, Territo MC, et al. Induction of endothelial expression of granulocyte and macrophage colony-stimulating factors by modified low density lipoproteins. Nature 1990;344:254–7.
66. Kume N, Cybulsky M, Gimbrone MA Jr. Lysophosphatidyl-choline, a component of atherogenic lipoproteins, induces mononuclear leukocyte adhesion molecules in cultured endothelial cells. J Clin Invest 1992;90:1138–44.
67. Hessler JR, Robertson AJ, Chisolm GM. LDL-induced cytotoxicity and its inhibition by HDL in human vascular smooth muscle and endothelial cells in culture. Atherosclerosis 1979;32:213–29.
68. Parthasarathy S, Barnett J, Fong LG. High density lipoprotein inhibits the oxidative modification of low-density lipoprotein. Biochim Biophys Acta 1990;1044:275–83.
69. Stafforini DM, Zimmerman GA, McIntyre TM, Prescott SM. The platelet activating factor acetylhydrolase from human plasma prevents oxidative modification of low density lipoprotein. Trans Am Assoc Physicians 1993;106:44–63.
70. Resnick N, Collins T, Atkinson WJ, Bonthron DT, Dewey CFJ, Gimbrone MA Jr. Platelet-derived growth factor B chain promoter contains a *cis*-acting fluid shear-stress-responsive element. Proc Natl Acad Sci U S A 1993;90:4591–5.
71. Nagel T, Resnick N, Atkinson WJ, Dewey CFJ, Gimbrone MA Jr. Shear stress selectively upregulates intercellular adhesion molecule-1 expression in cultured human vascular endothelial cells. J Clin Invest 1994;94:885–91.
72. De Caterina R, Libby P, Peng H-B, et al. Nitric oxide decreases cytokine-induced endothelial activation. Nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. J Clin Invest 1995;96:60–8.
73. Shin W, Hong Y, Peng H, De Caterina R, Libby P, Liao JK. Nitric oxide attenuates vascular smooth muscle cell activation by interferon-γ. The role of constitutive NF-κB activity. J Biol Chem 1996;271:11317–24.
74. Peng H-B, Libby P, Liao JK. Induction and stabilization of I kappa B alpha by nitric oxide mediates inhibition of NF-kappa B. J Biol Chem 1995;270:14214–9.
75. Kromann N, Green A. Epidemiological studies in the Upernavik district, Greenland. Acta Med Scand 1980;208:401–6.
76. Bang HO, Dyerberg J, Hjorne N. The composition of food consumed by Greenland Eskimos. Acta Med Scand 1976;200:69–73.
77. Kagawa Y, Nishizawa M, Suzuki M. Eicosapolyenoic acid of serum lipids of Japanese islanders with low incidence of cardiovascular diseases. J Nutr Sci Vitaminol (Tokyo) 1982;28:441–53.
78. Hirai A, Hamazaki T, Terano T. Eicosapentaenoic acid and platelet function in Japanese. Lancet 1980;2:1132–3.
79. Arthaud B. Cause of death in 339 Alaskan natives as determined by autopsy. Arch Pathol 1970;90:433–8.
80. Middaugh JP. Cardiovascular deaths among Alaskan natives 1980–1986. Am J Public Health 1990;80:282–5.
81. Kromhout D, Bosschieter EB, De Lezenne Coulander C. The inverse relation between fish consumption and 20-year mortality from coronary heart disease. N Engl J Med 1985;312:1205–9.
82. Shekelle R, Missel L, Paul O, MacMillan-Schryock A, Stamler J. Fish consumption and mortality from coronary heart disease. N Engl J Med 1985;313:820 (letter).
83. Soei L, Lamers J, Sassen L, et al. Fish oil: a modulator of experimental atherosclerosis in animals. In: Kristensen S, Endres S, De Caterina R, Schmidt E, eds. n−3 Fatty acids: prevention and treatment in vascular disease. London: Springer, 1995:77–84.
84. Newman WP, Middaugh JP, Propst MT, Rogers DR. Atherosclerosis in Alaska natives and non-natives. Lancet 1993;341:1056–7.
85. Eritsland J, Arnesen H, Grønseth K, Field NB, Abdelnoor M. Effect of supplementation with n−3 fatty acids on graft patency in patients undergoing coronary artery bypass operation. Results from the SHOT study. Eur Heart J 1994;15:29 (abstr).
86. De Caterina R, Cybulsky MI, Clinton SK, Gimbrone MA Jr, Libby P. The omega-3 fatty acid docosahexaenoate reduces cytokine-induced expression of pro-atherogenic and pro-inflammatory proteins in human endothelial cells. Arterioscler Thromb 1994;14:1829–36.
87. De Caterina R, Cybulsky MA, Clinton SK, Gimbrone MA Jr, Libby P. Omega-3 fatty acids and endothelial leukocyte adhesion molecules. Prostaglandins Leukot Essent Fatty Acids 1995;52:191–5.
88. Baker RR, Chang H-Y. The hydrolysis of natural phosphatidylethanolamines by phospholipase A2 from rat serum: a degree of selectivity is shown for docosahexaenoate release. Biochim Biophys Acta 1992;1125:56–61.
89. Van Snick J. Interleukin-6: an overview. Annu Rev Immunol 1990;8:253–78.
90. Weber C, Erl W, Pietsch A, Danesch U, Weber P. Docosahexaenoic acid selectively attenuates induction of vascular cell adhesion molecule-1 and subsequent monocytic cell adhesion to human endothelial cells stimulated by tumor necrosis factor-alpha. Arterioscler Thromb Vasc Biol 1995;15:622–8.
91. De Caterina R, Bernini W, Carluccio MA, Liao JK, Libby P. Structural requirements for inhibition of cytokine-induced endothelial activation by unsaturated fatty acids. J Lipid Res 1998;39:1062–70.
92. Carluccio MA, Massaro M, Bonfrate C, et al. Oleic acid inhibits endothelial activation: a direct vascular antiatherogenic mechanism of a nutritional component in the Mediterranean diet. Arterioscler Thromb Vasc Biol 1999;19:220–8.
93. Meskini N, Nemoz G, Chapuy P, et al. Glutathione peroxidase activity and metabolism of arachidonic acid in peripheral blood mononuclear cells from elderly subjects. Clin Sci 1993;85:203–11.
94. Endres S, Ghorbani R, Kelley VE, et al. The effect of dietary supplementation with n−3 polyunsaturated fatty acids on the synthesis of interleukin-1 and tumor necrosis factor by mononuclear cells. N Engl J Med 1989;320:265–71.
95. Hansen JB, Olsen JO, Wilsgard L, Østerud B. Effects of dietary supplementation with cod liver oil on monocyte thromboplastin synthesis, coagulation and fibrinolysis. J Intern Med Suppl 1989;225:133–9.
96. Kaminski WE, Jendraschak E, Kiefl R, von Schacky C. Dietary omega-3 fatty acids lower levels of platelet-derived growth factor mRNA in human mononuclear cells. Blood 1993;81:1871–9.
97. Lehr A-H, Hübner C, Finckh B, et al. Dietary fish oil reduces leukocyte/endothelium interaction following systemic administration of oxidatively modified low density lipoproteins. Circulation 1991;84:1725–31.
98. Maier JA, Hla T, Maciag T. Cyclooxygenase is an immediate-early gene induced by interleukin-1 in human endothelial cells. J Biol Chem 1990;265:10805–8.
99. Habib A, Creminon C, Frobert Y, Grassi J, Pradelles P, Maclouf J. Demonstration of an inducible cyclooxygenase in human endothelial cells using antibodies raised against the carboxyl-terminal region of the cyclooxygenase-2. J Biol Chem 1993;268:23448–54.
100. Hla T, Neilson K. Human cyclooxygenase-2 cDNA. Proc Natl Acad Sci U S A 1992;89:7384–8.
101. De Caterina R, Bernini W, Carluccio MA, Liao JK, Libby P. Structural requirements for inhibition of cytokine-induced endothelial activation by unsaturated fatty acids. J Lipid Res 1998;39:1062–70.

EXHIBIT 4

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Dietary Supplementation With Marine Omega-3 Fatty Acids Improve Systemic Large Artery Endothelial Function in Subjects With Hypercholesterolemia

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OBJECTIVE This work was undertaken to determine whether dietary supplementation with marine omega-3 fatty acids improve systemic large artery endothelial function in subjects with hypercholesterolemia.

BACKGROUND Marine omega-3 fatty acids improve vascular function, but the underlying mechanism(s) are unclear. We studied the effects of marine omega-3 fatty acids on large artery endothelial function in subjects with hypercholesterolemia.

METHODS Hypercholesterolemic subjects with no other known cause for endothelial dysfunction were recruited to a prospective, placebo-controlled, randomized, double-blind, parallel-group study. Treatment with omega-3 fatty acids at a dose of 4 g/day (n = 15/group) was compared with placebo, at the beginning (day 0) and end (day 120) of a four-month treatment period. Endothelial function was assessed pre- and posttreatment by noninvasive ultrasonic vessel wall tracking of brachial artery flow-mediated dilation (FMD).

RESULTS Treatment with marine omega-3 fatty acids resulted in a significant improvement in FMD (0.05 ± 0.12 to 0.12 ± 0.07 mm, $p < 0.05$) and a significant reduction in triglycerides (2.07 ± 1.13 to 1.73 ± 0.95 mmol/liter, $p < 0.05$), whereas treatment with placebo resulted in no change in FMD (0.03 ± 0.10 to 0.04 ± 0.10 mm) or triglycerides (2.29 ± 2.09 to 2.05 ± 1.36 mmol/liter) (both $p < 0.05$ treated compared with control). Responses to sublingual glyceryl trinitrate were unchanged.

CONCLUSIONS Marine omega-3 fatty acids improve large artery endothelium-dependent dilation in subjects with hypercholesterolemia without affecting endothelium-independent dilation. (J Am Coll Cardiol 2000;35:265–70) © 2000 by the American College of Cardiology

Atherosclerotic coronary artery disease is a major cause of morbidity and mortality in Western civilization. Atherosclerosis may be regarded as the long-term consequence of a chronic inflammatory condition of large arteries (1), in which endothelial dysfunction (2) plays a key role. Endothelial function has been assessed in the coronary circulation by measuring vascular reactivity to intracoronary infusion of endothelium-dependent agonists such as acetylcholine. Patients with atherosclerotic coronary artery disease exhibit paradoxical vasoconstriction (3), as do patients with hypercholesterolemia and angiographically normal coronaries (4). Such tests are invasive, expensive and not without risk. An alternative approach is to study vascular reactivity noninvasively with ultrasonic assessment of brachial artery flow-mediated dilation (FMD) (5). The brachial artery is of a similar size to coronary arteries, and although it is unusual for the brachial artery to have significant atheroma, brachial responses have been shown to correlate well with responses in the coronary circulation for a given individual (6). Endothelial function can be measured noninvasively by "wall tracking" of brachial artery dilation in response to increased flow generated by hyperemia of the hand (7,8). Flow-related endothelial function has been shown to be mediated by NO (9,10) and impaired with all known risk factors for atheroma (11–15). Endothelial dysfunction, thus measured, appears to be representative of generalized endothelial dysfunction and offers a potentially useful measure of the susceptibility to atheroma.

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Abbreviations and Acronyms

DHA = docosahexanoic acid
EPA = eicosapentanoic acid
FMD = flow-mediated dilation
GTN = glyceryl trinitrate
HDL = high-density lipoprotein
LDL = low-density lipoprotein
NO = nitric oxide
RF = radio frequency
VLDL = very low density lipoprotein

Among dietary interventions that might protect against atheroma and its complications are diets rich in fish. The protective effects of diets rich in fish oil are quite strongly supported by experimental (16,17), epidemiological (18–23) and clinical trial data (24). Beneficial effects of fish oil supplementation on endothelial function in resistance arteries in vivo (25) and in vitro (26) have been reported. Accordingly, we carried out a placebo-controlled four-month trial of marine omega-3 fatty acids ("fish oil") in fit subjects with hypercholesterolemia in order to test whether omega-3 fatty acids also improved large artery endothelial function as measured by flow-mediated brachial artery dilation.

METHODS

Subjects. Thirty subjects were recruited from the Lipid Clinic at the University Hospital of Wales, Cardiff. All had confirmed hypercholesterolemia (serum total cholesterol >6.5 mmol/liter) after a low-fat diet for three months. Those already on lipid-lowering agents had stable cholesterol levels, and the dose of lipid-lowering agent remained unaltered for the duration of the trial. Exclusion criteria were active smokers, recent ex-smokers (two years), diabetes, hypertension (including treated hypertension) and a clinical history of coronary, cerebral or peripheral vascular disease. Subjects taking hormone supplements, vasoactive medications or proprietary medications such as vitamins, antioxidants or fish oils were also excluded.

Study design. This was a prospective, placebo-controlled, randomized, double-blind, parallel-group trial. Effects of treatment were compared at the beginning (day 0) and end (day 120) of a four-month treatment period in parallel groups of subjects with hypercholesterolemia. All subjects underwent a full clinical examination. Venous blood samples were obtained, and flow-mediated brachial artery dilation was measured on days 0 and 120.

Marine omega-3 fatty acids. Thirty subjects were recruited and randomly assigned to two groups of 15 subjects to receive: a) marine omega-3 fatty acids (K85; Pronova a.s, Oslo, Norway), or b) placebo (corn oil), each as two 1-g capsules twice daily for 120 days. Baseline characteristics were similar in both groups (Table 1). The K85 capsules used in the study were omega-3 concentrate enriched in eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) and produced from high-quality whole-body fish oil. The concentration of these two fatty acids as esters is about 85%, which is approximately threefold higher than in Maxepa capsules. K85 also contains 4 IU of vitamin E per capsule. The corn oil placebo capsules contained no vitamin E.

Table 1. Baseline Characteristics of Subjects in Omega-3 Fatty Acids Study

	Placebo (n = 15)	Marine Omega-3 Fatty Acid (n = 13)
Age (years)	50 ± 12	56 ± 13
Male/Female (n)	11/4	8/6
Total cholesterol (mmol/liter)	7.45 ± 0.64	7.85 ± 1.64
HDL cholesterol (mmol/liter)	1.34 ± 0.39	1.41 ± 0.38
LDL cholesterol (mmol/liter)	5.31 ± 0.73	5.00 ± 0.73
Triglyceride (mmol/liter)	2.29 ± 2.09	2.07 ± 1.13
Glucose (mmol/liter)	4.95 ± 0.66	4.95 ± 0.59
Body mass index (kg/m^2)	25.18 ± 2.70	26.90 ± 3.04
Smoking status	0	0
Blood pressure	137 ± 10	138 ± 13
mm Hg	86 ± 9	87 ± 9
Statin (n)	3	1
Fibrate (n)	1	2
Nil (n)	12	10

No significant differences between the two groups. Data are given as mean ± SD.

Measurement of endothelial function. Flow-mediated dilation was measured by ultrasonic wall tracking as reported previously (7). The system used comprises a specially adapted duplex color flow echo machine (Diasonics Spectra) with a 7.5-MHz linear phased-array transducer (giving high axial resolution), a personal computer and a 4-Mb high-speed memory. The brachial artery is identified using the ultrasound transducer, and anatomical landmarks are identified to allow repeat studies. A standoff device containing ultrasound-coupling gel prevents compression of the anterior wall of the artery. The transducer is held in a stereotactic clamp, and a two-dimensional longitudinal B-mode image of the brachial artery is obtained. The radio frequency (RF) signals (sampling frequency 1 kHz) from the M-mode output are digitized and relayed to the wall tracking system (Vadirec, Medical Systems Arnhem, Oosterbeek, The Netherlands). On completion of 10-s data acquisition, the RF signal is displayed so that the position of the anterior and posterior vessel walls on the RF signal can be identified and marked. Vessel wall movements are tracked using the stored RF signals to produce displacement waveforms of the anterior and posterior vessel walls together with the distension waveform (diameter change as a function of time). The distension waveform enables measurement of "end-

diastolic" diameter for each beat (theoretical resolution ± 3 μm) (27).

Blood pressure was recorded throughout the study by photo-plethysmography (Finapres) from a finger cuff on the middle finger of the ipsilateral arm. Blood flow was measured throughout the study using an 8-MHz continuous wave Doppler probe mounted at an angle of 60° in a perspex block and positioned over the brachial artery distal to the 7.5-MHz probe. The Doppler signals were analyzed by a spectrum analyser (SciMed Dopstation, Bristol, UK) and stored on metal audiotape using a high-performance recorder (Nakamichi B-100E, Nakamichi Corporation, Japan). Brachial artery blood flow was calculated by multiplying the mean blood velocity (corrected for Doppler angle) by the internal brachial artery diameter measured by wall tracking.

Study protocol. All studies were performed in the morning in a temperature-controlled room (21°C to 23°C) on fasting subjects after a 15-min supine rest, with the arm held outstretched on a pneumatic cushion. Patients were asked to avoid caffeine-containing beverages for 12 h before the study. Measurements were made at baseline, during hand hyperemia and after sublingual glyceryl trinitrate (GTN), an endothelium-independent vasodilator.

Hand hyperemia. A pediatric sphygmomanometer cuff was inflated at the wrist to suprasystolic pressure (systolic pressure ± 50 mm Hg) for 5 min. Blood flow was recorded from 15 s before until 90 s after cuff release, and internal brachial artery diameter was measured for 10 s at 60 to 70 s after cuff release. All measurements were repeated ≥ 15 min later until values reached original baseline levels. Flow-mediated dilation was defined as brachial artery diameter at 60 to 70 s after cuff release minus baseline diameter (expressed in mm).

GTN. Measurements were repeated 3 min after sublingual GTN spray (400 μg).

Serum concentrations. Fasting venous blood samples were obtained at each study for measurement of total serum cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, very low density lipoprotein (VLDL) cholesterol (calculated), triglyceride and glucose. Urea and electrolytes and liver function were also measured.

Statistics. Data are presented as mean ± SD. Data were tested for normality using the Shapiro-Wilks test. Where normality was established unpaired Student's *t* tests were used to make comparisons between groups and Student's paired *t* test to make comparisons within groups. $p < 0.05$ was regarded as significant.

Ethical approval. Ethical approval for this study was granted by the local research and ethics committee of South Glamorgan Health Authority. All subjects gave written informed consent. The investigation conformed to the principles outlined in the Declaration of Helsinki.

RESULTS

There were no significant differences between the treatment groups at baseline (Table 1). Of the 15 subjects allocated to each group, 28 completed the study. The two subjects who did not return for the second scan were in the fish oil group; no reasons for leaving the study were reported. No adverse side effects were reported. Compliance with treatment was assessed by a count of capsules returned at study end and was considered satisfactory (>95% for placebo and marine omega-3 fatty acids).

Changes in brachial artery blood flow immediately after wrist cuff release (peak flow, 1 min after cuff release and 3 min after sublingual GTN) were similar in all groups before and after treatment (Fig. 1A). Flow-mediated dilation increased significantly after omega-3 fatty acids treatment compared with placebo (Table 2, Fig. 1B). Treatment with omega-3 fatty acids significantly reduced triglyceride levels (Table 2) but had no effect on serum concentration of total cholesterol, VLDL, LDL or HDL cholesterol, whereas treatment with placebo had no significant effect on the lipid profiles (Table 2).

There was no correlation between the improvement in endothelium-dependent FMD and the reduction in triglycerides in the marine omega-3 fatty acids group.

Glyceryl trinitrate-induced dilation was similar pre- and posttreatment in both groups (Table 2, Fig. 1C).

DISCUSSION

The major findings of this double-blind placebo-controlled study are that marine omega-3 fatty acids (fish oils) improve endothelial function in systemic large arteries in patients with hypercholesterolemia. This study also confirms the loss of FMD in the brachial artery, reflecting impaired endothelium-derived nitric oxide (NO) activity in hypercholesterolemic patients as previously reported (28,29). It confirms also that vascular smooth muscle dilator responsiveness to NO is preserved, as evidenced by the normal dilator response to GTN.

Dietary supplementation with marine omega-3 fatty acids for four months resulted in a significant improvement in endothelium-dependent FMD of the brachial artery. This artery is of a similar size to the coronary arteries, and brachial responses have been shown to correlate well with responses in the coronary circulation for a given individual (6). There was a significant decrease in serum triglycerides with omega-3 fatty acids supplementation, which has previously been reported (30). In this study, the improvement in endothelial function did not correlate with the reduction in triglycerides, which is not unexpected, as our subjects had hypercholesterolemia with significantly elevated total and LDL cholesterol levels, with only modestly elevated triglyceride levels. Hypertriglyceridemia is not as strongly associ-

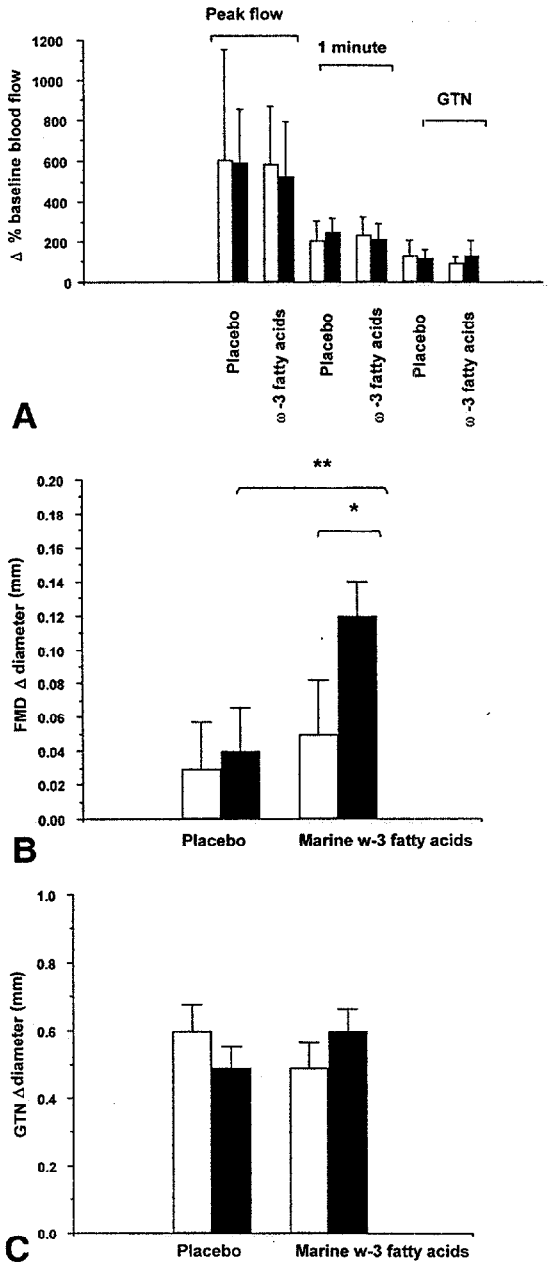

Figure 1. The effect of four months of treatment with placebo and omega-3 fatty acids on the following. **A**, Brachial artery blood flow (expressed as percent change from baseline) at: peak flow immediately after wrist cuff deflated, 1 min after cuff deflated and 3 min after 400 μg sublingual GTN. There were no significant differences between groups. **B**, Flow-mediated dilation expressed as absolute change (mm) from baseline diameter. There is a significant improvement within the omega-3 fatty acids group posttreatment (*$p < 0.05$) and when compared with placebo posttreatment (**$p < 0.05$). **C**, Glyceryl trinitrate-mediated dilation expressed as absolute change (mm) from baseline diameter. There were no significant differences between groups. Data are presented as mean ± SEM.
Solid bars = posttreatment; **Open bars** = pretreatment.

ated with coronary atherosclerosis (31) as elevated LDL cholesterol (32), and when multivariate analysis is used to correct for LDL and HDL cholesterol, much of the association with hypertriglyceridemia disappears (33). Further support for a lack of association between hypertriglyceridemia and endothelial function comes from a recent study that demonstrated that severe hypertriglyceridemia was not associated with significant dysfunction of the L-arginine/NO pathway in forearm resistance vessels (34).

Possible mechanism(s) for improvement in endothelial function. The mechanism underlying the improvement in endothelial function in patients treated with marine omega-3 fatty acids in this study is unclear. We did not give indomethacin, therefore, we cannot exclude the possibility that vasodilator prostaglandins played a role. However, based on animal studies (16) and a recent clinical study (26), this would appear to be unlikely. Recent evidence suggests that the mechanism(s) responsible for the benefit seen with a fish oil-rich diet is likely to relate to changes in membrane bilipid layer composition with multiple potential effects on endothelial function. The recent in vitro small artery study by Goode et al. (26) demonstrated that the greatest improvement in endothelial function occurred in those patients who had the greatest increase in membrane EPA and DHA, as reflected by increases in these fatty acids in the red cell membrane. Hence, it is possible that dietary supplementation with marine omega-3 fatty acids may change the membrane fluidity of endothelial cells, promoting increased synthesis and/or release of NO. The marine omega-3 fatty acids preparation we used contains a small amount of antioxidant vitamin E (equal to approximately 16 IU vitamin E/day), which theoretically may be expected to have an effect. In a study of similar design, we have shown dietary supplementation with 20 IU vitamin E/day for four months had no benefit in hypercholesterolemic subjects (unpublished data, J.G.), as have others (35). Feeding humans fish oils has been shown to reduce oxygen-derived free radical formation in neutrophils and monocytes, and to enhance NO production by cultured human endothelial cells (36). Speculatively, it is also possible that a reduction in the formation of oxygen derived free radicals by endothelial cells and thus increased bioavailability of NO contributed to the effects observed in this study.

Clinical implications. This study adds evidence relevant to the complex but important issue of dietary reduction of atherogenesis, given the key-initiating role of endothelial dysfunction. Marine omega-3 fatty acids offer attractive potential, which has recently been supported by evidence of clinical benefit (37). Clinical evidence is necessarily weighted towards changes in the end-stage complications of coronary artery disease, and this may be susceptible to measures that alter plaque cap inflammation and vulnerability to fissuring and thrombosis as well as the intrinsic process of atherogenesis. The latter changes may be longer term and less readily detectable. This study, through the measurement of endothelial function indices, supports a possible future role for omega-3 fatty acids and may help to

Table 2. Effects of Four Months of Treatment With Placebo and Omega-3 Fatty Acids

	Placebo (n = 15)		Omega-3 Fatty Acids (n = 13)	
	Pretreatment	Posttreatment	Pretreatment	Posttreatment
Baseline diameter (mm)	4.17 ± 0.65	4.08 ± 0.68	4.12 ± 0.84	4.02 ± 0.77
Absolute change in baseline diameter				
FMD (mm)	0.03 ± 0.10	0.04 ± 0.10	0.05 ± 0.12	0.12 ± 0.07*
GTN 400 μg (mm)	0.60 ± 0.29	0.49 ± 0.23	0.49 ± 0.25	0.60 ± 0.22
Total cholesterol (mmol/liter)	7.45 ± 0.64	7.20 ± 0.71	7.85 ± 1.64	7.69 ± 1.25
HDL cholesterol (mmol/liter)	1.34 ± 0.39	1.28 ± 0.29	1.41 ± 0.38	1.34 ± 0.38
LDL cholesterol (mmol/liter)	5.31 ± 0.73	5.25 ± 0.71	5.00 ± 0.73	5.35 ± 1.02
Triglyceride (mmol/liter)	2.29 ± 2.09	2.05 ± 1.36	2.07 ± 1.13	1.73 ± 0.95*
Glucose (mmol/liter)	4.95 ± 0.66	4.82 ± 0.40	4.95 ± 0.59	5.05 ± 0.35

Flow-mediated dilation (FMD) and GTN-induced dilation are shown as absolute change compared with baseline diameter. Flow-mediated dilation was significantly increased and triglyceride level decreased in the omega-3 fatty acids group compared with placebo (both *$p < 0.05$).

explain the benefits shown in epidemiological studies of populations consuming a fish-rich diet.

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REFERENCES

1. Berliner JA, Navab M, Fogelman AM, et al. Atherosclerosis: basic mechanisms oxidation, inflammation, and genetics. Circulation 1995; 91:2488–96.
2. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990's. Nature 1993;362:801–9.
3. Ludmer PL, Selwyn AP, Shook TL, et al. Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries. N Engl J Med 1986;315:1046–51.
4. Leung WH, Lau CP, Wong CK. Beneficial effect of cholesterol-lowering therapy on coronary endothelium-dependent relaxation in hypercholesterolemic patients. Lancet 1993;341:1496–500.
5. Celermajer DS, Sorenson KE, Gooch VM, et al. Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. Lancet 1992;340:1111–5.
6. Anderson T, Uehata A, Gerhard M, et al. Close relation of endothelial function in the human coronary and peripheral circulations. J Am Coll Cardiol 1995;26:1235–41.
7. Ramsey MW, Goodfellow J, Jones CJH, Luddington LA, Lewis MJ, Henderson AH. Endothelial control of arterial distensibility is impaired in chronic heart failure. Circulation 1995;92:3212–9.
8. Ramsey MW, Goodfellow J, Bellamy MF, Lewis MJ, Henderson AH. Non-invasive detection of endothelial dysfunction. Lancet 1996;348: 128–9.
9. Rubanyi GM, Romero JC, Vanhoutte PM. Flow-induced release of endothelium-derived relaxing factor. Am J Physiol 1986;250: H1145–9.
10. Joannides R, Haefeli WE, Linder L, et al. Nitric oxide is responsible for flow-dependent dilation of human peripheral conduit arteries in vivo. Circulation 1995;91:1314–9.
11. Celermajer DS, Sorenson KE, Bull C, Robinson J, Deanfield JE. Endothelium-dependent dilation in the systemic arteries of asymptomatic subjects relates to coronary risk factors and their interaction. J Am Coll Cardiol 1994;24:1468–74.
12. Vita JA, Treasure CB, Ganz P, Cox DA, Fish RD, Selwyn AP. Atherosclerosis impairs flow-mediated dilation of coronary arteries. Circulation 1989;80:458–65.
13. Celermajer DS, Sorensen KE, Georgakopoulos D, et al. Cigarette smoking is associated with dose-related and potentially reversible impairment of endothelium-dependent dilation in healthy young adults. Circulation 1993;88:2148–55.
14. Clarkson P, Celermajer DS, Donald AE, et al. Impaired vascular reactivity in insulin-dependent diabetes mellitus is related to disease duration and low density lipoprotein cholesterol levels. J Am Coll Cardiol 1996;28:573–9.
15. Goodfellow J, Ramsey MW, Luddington LA, et al. Endothelium and inelastic arteries: an early marker of vascular dysfunction in non-insulin dependent diabetes. Br Med J 1996;312:744–5.
16. Shimokawa H, Lam JYT, Chesebro JH, Bowie EJW, Vanhoutte PM. Effects of dietary supplementation with cod-liver oil on endothelium-dependent responses in porcine coronary arteries. Circulation 1987;76: 898–905.
17. Shimokawa H, Vanhoutte PM. Dietary cod-liver oil improves endothelium-dependent responses in hypercholesterolemic and atherosclerotic porcine coronary arteries. Circulation 1988;78:1421–30.
18. Kromhout D, Bosschieter EB, de Lezenne Coulander C. The inverse relation between fish consumption and 20 year mortality from coronary heart disease. N Engl J Med 1985;312:1205–9.
19. Vollset SE, Heuch I, Bjelke E. Fish consumption and mortality from coronary heart disease. N Engl J Med 1985;313:821–2.
20. Curb JD, Reed DM. Fish consumption and mortality from coronary heart disease. N Engl J Med 1985;313:821–2.
21. Shekelle RB, Missell L, Paul O, Shyrock AM, Stamler J. Fish consumption and mortality from coronary heart disease. N Engl Med J 1985;313:820.
22. Ascherio A, Rimm EB, Stampfer MJ, Giovannucci EL, Willett WC. Dietary intake of marine nomega-3 fatty acids, fish intake and the risk of coronary disease among men. N Engl J Med 1995;332: 977–82.
23. Kromann N, Green A. Epidemiological studies in the Upernavik district, Greenland: incidence of some chronic diseases. Acta Med Scand 1980;208:401–6.
24. Burr ML, Fehily AM, Gilbert JF, et al. Effects of changes in fat, fish, and fibre intakes on death and myocardial reinfarction: diet and reinfarction trial (DART). Lancet 1989;2:75.
25. Chin JPF, Dart AM. Therapeutic restoration of endothelial function in hypercholesterolemic subjects: effect of fish oils. Clin Exp Pharmacol Physiol 1994;21:749–55.
26. Goode GK, Garcia S, Heagerty AM. Dietary supplementation with marine fish oil improves *in vitro* small artery endothelial function in hypercholesterolemic patients: a double-blind placebo-controlled study. Circulation 1997;96:2802–7.

27. Hoeks APG, Brands PJ, Smeets FAM, Reneman RS. Assessment of the distensibility of superficial arteries. Ultrasound Med Biol 1990;16: 121–8.
28. Sorenson KE, Celermajer DS, Georgakopoulos D, Hatcher G, Betteridge J, Deanfield JE. Impairment of endothelium-dependent dilation is an early event in children with familail hypercholesterolemia, and is related to the Lp(a) level. J Clin Invest 1994;93:50–5.
29. Goodfellow J, Ramsey MW, Luddington LA, Jones CJH, et al. Flow related endothelial function and serum cholesterol- non-invasive markers of atherogenesis. Circulation 1994;90 Suppl I:573.
30. Harris WS. Fish oils and plasma lipid and lipoprotein metabolism in humans: a critical review. J Lipid Res 1989;30:785–807.
31. Castelli WP. The triglyceride issue: a view from Framingham. Am Heart J 1986;112:432–7.
32. Stampfer MJ, Sacks FM, Salvini S, Willet WC, Hennekens CH. A prospective study of cholesterol, apolipoproteins, and the risk of myocardial infarction. N Eng J Med 1991;325:373–81.
33. Austin MA. Epidemiological association between hypertriglyceridaemia and coronary heart disease. Semin Thromb Haemost 1988;14: 137–42.
34. Chowienczyk PJ, Watts GF, Wierzbicki AS, Cockcrot JR, Brett SE, Ritter JM. Preserved endothelial function in patients with severe hypertriglyceridemia and low functional lipoprotein lipase activity. J Am Coll Cardiol 1997;29:964–8.
35. McDowell IF, Brennan GM, McEneny J, et al. The effect of probucol and vitamin E treatment on the oxidation of low-density lipoprotein and forearm vascular responses in humans. Eur J Clin Invest 1994;24: 759–65.
36. Okuda Y, Kawashima K, Sawada T, et al. Eicosapentanoic acid enhances nitric oxide production by cultured human endothelial cells. Biochem Biophys Res Comm 1997;232:487–91.
37. Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarcto Miocardico. Lancet 1999;354:447–55.

EXHIBIT 5

Polyunsaturated fatty acids in the food chain in the United States[1,2]

PM Kris-Etherton, Denise Shaffer Taylor, Shaomei Yu-Poth, Peter Huth, Kristin Moriarty, Valerie Fishell, Rebecca L Hargrove, Guixiang Zhao, and Terry D Etherton

ABSTRACT In the United States, intake of n−3 fatty acids is ≈1.6 g/d (≈0.7% of energy), of which 1.4 g is α-linolenic acid (ALA; 18:3) and 0.1–0.2 g is eicosapentaenoic acid (EPA; 20:5) and docosahexaenoic acid (DHA; 22:6). The primary sources of ALA are vegetable oils, principally soybean and canola. The predominant sources of EPA and DHA are fish and fish oils. Intake data indicate that the ratio of n−6 to n−3 fatty acids is ≈9.8:1. Food disappearance data between 1985 and 1994 indicate that the ratio of n−6 to n−3 fatty acids has decreased from 12.4:1 to 10.6:1. This reflects a change in the profile of vegetable oils consumed and, in particular, an approximate 5.5-fold increase in canola oil use. The ratio of n−6 to n−3 fatty acids is still much higher than that recommended (ie, 2.3:1). Lower ratios increase endogenous conversion of ALA to EPA and DHA. Attaining the proposed recommended combined EPA and DHA intake of 0.65 g/d will require an approximately 4-fold increase in fish consumption in the United States. Alternative strategies, such as food enrichment and the use of biotechnology to manipulate the EPA and DHA as well as ALA contents of the food supply, will become increasingly important in increasing n−3 fatty acid intake in the US population. *Am J Clin Nutr* 2000;71(suppl): 179S–88S.

KEY WORDS Highly unsaturated fatty acids, α-linolenic acid, eicosapentaenoic acid, docosahexaenoic acid, n−6 fatty acids, n−3 fatty acids

INTRODUCTION

Dietary recommendations continue to evolve as we gain a better understanding of the health effects of nutrients. In recent years, the optimal level of n−3 fatty acids in the diet has been the focal point of intense scientific scrutiny. This has resulted in recommendations to increase consumption of the highly unsaturated n−3 fatty acids, specifically eicosapentaenoic acid (EPA; 20:5n−3) and docosahexaenoic acid (DHA; 22:6n−3), from 0.1–0.2 to 0.65 g/d. To achieve this 4-fold increase in intake, it is important to know what the current intake is, what the food sources are in the diet, and how the diet can be modified to appreciably increase the intake of these highly unsaturated fatty acids (HUFAs; fatty acids with ≥20 carbon atoms and ≥4 double bonds). An increase of the HUFA consumption would likely compliment the current dietary recommendations to reduce saturated fatty acid intake and bring us closer to our long-sought goal of defining the ideal fatty acid profile of a diet that achieves the optimum health benefits.

This article will discuss the consumption practices and sources of n−3 fatty acids in the US diet. A major emphasis will be how, in practice, Americans can achieve recommended intakes of n−3 fatty acids. Approaches for increasing n−3 fatty acid intake include increasing the consumption of food sources of these fatty acids and using biotechnology to change the fatty acid composition of foods commonly eaten. Another consideration is that n−3 fatty acid supplements may be a useful alternative approach to achieving a pharmacologic intake. Irrespective of the approach taken to increase n−3 fatty acid intake, it is likely that during the next decade we will witness an increase in the consumption of n−3 fatty acids and thereby realize the health benefits they uniquely confer.

CONSUMPTION OF POLYUNSATURATED FATTY ACIDS IN THE US

Polyunsaturated fatty acids (PUFAs) contribute ≈7% of total energy intake and 19–22% of energy intake from fat in the diets of adults, a level that is within recommended intakes for both men and women. Linoleic acid (18:2n−6) is the major PUFA, comprising 84–89% of the total PUFA energy, whereas α-linolenic acid (ALA; 18:3n−3) contributes 9–11% of the total PUFA energy (equivalent to 1.1–1.6 g/d) in the diets of the adult population (**Table 1**).

The n−6 fatty acids, 18:4n−6 and 20:4n−6 (arachidonic acid), provide ≤0.1% of energy intake. In addition, EPA and DHA together provide ≤0.1–0.2% of energy intake. When expressed in grams in addition to percentage of PUFA energy intake, EPA and DHA provide ≤0.2 g/d and <2% of energy from PUFAs. Thus, it is evident that HUFAs do not contribute appreciably to fat intake (Table 1).

SOURCES OF n−3 FATTY ACIDS IN THE US DIET

Vegetable oils, fish, and plant sources

The predominant sources of n−3 fatty acids in the diet are vegetable oils and fish. Fish are the major source of EPA and DHA, whereas vegetable oils are the major source of ALA. Other

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TABLE 1
Polyunsaturated fatty acid intake of adults in the United States by sex and age[1]

	Total		18:2n−6		18:3n−3		18:4n−3 + 20:4n−6		20:5n−3 + 22:6n−3	
Sex and age (y)	1987–1988	1989–1991	1987–1988	1989–1991	1987–1988	1989–1991	1987–1988	1989–1991	1987–1988	1989–1991
					g/d					
Males										
12–19	16 ± 0.3	18 ± 1.1	14 ± 0.3	16 ± 0.9	2 ± 0.03	2 ± 0.07	0.1 ± 01	0.1 ± 01	0.1 ± 0.01	0.1 ± 0.07
20–39	17 ± 0.2		15 ± 0.2		2 ± 0.02		0.1 ± 01		0.1 ± 0.01	
20–49		18 ± 0.4		16 ± 0.4		2 ± 0.03		0.1 ± 01		0.2 ± 0.01
>40	16 ± 0.2		14 ± 0.2		2 ± 0.02		0.1 ± 01		0.1 ± 0.01	
50–69		16 ± 0.5		15 ± 0.4		1 ± 0.03		0.1 ± 01		0.2 ± 0.03
Females										
12–19	12 ± 0.3	13 ± 0.4	11 ± 0.2	12 ± 0.4	1 ± 0.03	1 ± 0.03	0.1 ± 0	0.1 ± 01	0.1 ± 0	0.1 ± 0.02
20–39	12 ± 0.2		11 ± 0.1		1 ± 0.01		0.1 ± 0		0.1 ± 0	
20–49		13 ± 0.2		11 ± 0.2		1 ± 0.02		0.1 ± 01		0.1 ± 0.01
>40	11 ± 0.1	12 ± 0.3	10 ± 0.1		1 ± 0.01		0.1 ± 0		0.1 ± 0	
50–69				11 ± 0.3		1 ± 0.02		0.1 ± 01		0.2 ± 0.02
Males and females										
>70		12 ± 0.3		11 ± 0.3		1 ± 0.02		0.1 ± 01		0.2 ± 0.00

[1] $\bar{x}$ ± SE of 3-d intakes. Data for 1987–1988 adapted from reference 1. Data for 1989–1991 adapted from reference 2.

sources include nuts and seeds, vegetables and some fruit, and egg yolk, poultry, and meat, all of which collectively contribute minor quantities of n−3 fatty acids to the diet.

Of the commonly consumed oils in the United States, soybean and canola oil are the primary sources of ALA. The contents of ALA in soybean and canola oil are ≈7.8% and 9.2%, respectively. Flaxseed oil is a particularly rich source of n−3 fatty acids (ie, ALA) although it is not a commonly used food oil.

Some fatty fish, most notably halibut, mackerel, herring, and salmon, are rich sources of EPA and DHA. For example, salmon contains 1.0–1.4 g n−3 fatty acids/100 g edible portion (raw), whereas mackerel contains ≈2.5 g n−3 fatty acids (**Table 2**). Interestingly, and importantly, the content of n−3 fatty acids can vary appreciably among different types of fish. Specifically, Atlantic, Coho, and Sockeye salmon have markedly higher amounts of EPA and DHA than does Chinook salmon. Other lean varieties of fish do provide n−3 fatty acids and are sources of EPA and DHA, but to a much lesser extent.

In the United States, DHA per capita disappearance from fish is 0.25 g/d, which is similar to the worldwide average of 0.23 g/d; that of EPA is 0.16 g/d, compared with a worldwide average of 0.15 g/d. In the United States, DHA and EPA provide, on average, ≈61% and 39% of HUFA intake, respectively.

Plant sources, ie, nuts, seeds, vegetables, legumes, grains, and fruit provide dietary ALA (**Table 3**). Of these specific foods, nuts, seeds, and soybeans are relatively rich sources of ALA. Because fats and oils contribute ≈87% of the ALA in the US diet (7), it is apparent that the contribution of other terrestrial sources is minor.

Purslane, a vegetable used in soups and salads along the Mediterranean basin and in the Middle East, is unique because it is the richest source of ALA of any green leafy vegetable examined (6, 8). Moreover, it is one of the few plants known to be a source of EPA. Although not typically consumed in the US diet, purslane is nonetheless found in all 50 states and certainly could be developed as an important source of dietary n−3 fatty acids.

Food group contributors of dietary n−3 fatty acids

Central to the discussion of identifying food sources of n−3 fatty acids in the US diet is also understanding which food groups are important contributors of ALA, EPA, and DHA. Jonnalagadda et al (1) reported the contribution of major food categories to the intake of individual fatty acids using dietary data collected in the Nationwide Food Consumption Survey (1987–1988). This study showed that for both males and females the food group "meat, poultry, fish, and mixtures" contributed ≈90% of the EPA and DHA in the diet, and that this largely reflected fish consumption. Eggs were also a source of EPA and DHA (**Table 4**). Several food categories were found to be important sources of ALA, but this was largely a reflection of the use of vegetable oils rich in ALA for the preparation of many foods in these categories. For example, "grain products," "vegetables," and "meat, poultry, fish, and mixtures" were the predominant contributors of ALA to the diet along with "fats, oils, and salad dressings." The data presented clearly show that to appreciably increase EPA and DHA in the diet, a parallel increase in fatty fish or fish oil consumption is required. Likewise, to increase ALA intake it will be necessary to increase consumption of vegetable oils high in ALA at the expense of other fats in the diet.

TABLE 2
n−3 Fatty acid content of selected seafood[1]

Seafood	n−3 Fatty acids
	% by wt
Mackerel	1.8–5.3
Herring	1.2–3.1
Salmon	1.0–1.4
Tuna	0.5–1.6
Trout	0.5–1.6
Halibut	0.4–0.9
Shrimp	0.2–0.5
Cod	0.2–0.3
Plaice	≈0.2
Flounder	≈0.2
Haddock	0.1–0.2

[1] Values are ranges unless indicated otherwise. The fatty acid content of seafood varies considerably by season and location of catch. Adapted from references 3 and 4.

n−3 Fatty acid supplements

A variety of n−3 fatty acid supplements are available to consumers. Many of these supplements are derived from marine oils and contain 180 mg EPA and 120 mg DHA per capsule. Another source of n−3 fatty acids is cod-liver oil in some capsules that contain 173 mg EPA and 120 mg DHA. However, these supplements must be taken with caution because of the high amounts of vitamin A and vitamin D in them. A vegetarian source of DHA (100 mg/capsule) derived from algae is now available.

Industry estimates indicate that ≈300 Mg (≈300 tons) of fish oil are used yearly for fish-oil supplements in the United States (I Newton, personal communication, 1998). On a per capita basis this is equivalent to 1.0 g fish oil/y. The average yearly contribution of EPA and DHA from fish-oil supplements to the US diet is 0.6–0.9 mg/person. Thus, fish-oil supplements currently are not an important source of HUFAs in the US diet.

VARIATION IN THE n−3 FATTY ACID COMPOSITION OF FOODS

There is considerable variation in n−3 fatty acid content of fish, vegetable oils, and animal products. This could significantly affect n−3 fatty acid consumption, thereby contributing appreciably to both intraindividual (within-person) and interindividual (between-person) variability in intake. Although there are no quantitative data about the intraindividual variability of n−3 intake, Basiotis et al (9) reported a relatively high intraindividual variability for total fat and linoleic acid intake based on food records of 29 individuals for 365 consecutive days. Interindividual variability of n−3 intake was reported by Dolecek and Grandits (10) on the basis of food intake records of 6438 men in the control group of the Multiple Risk Factor Intervention Trial. Interestingly, the SDs for EPA and DHA intake were larger than their corresponding means.

Another explanation for the high variability in n−3 fatty acid intake is the variation in the quantity of n−3 fatty acids found in fish. This is due primarily to variations in *1*) the diet, location, stage of maturity, sex, and size of the fish and the season and water temperature in which it was caught, and 2) the canning oils and preparation methods used (11, 12). Whether fish are farm-raised (ie, in aquaculture) or caught from the wild can affect their fatty acid composition because of the difference in nutrient composition of the diet.

Factors such as cultivar, variety, growing region, and climatic conditions have marked influences on the ALA content of soybean and canola oil. For example, there have been substantial efforts over the years to reduce the ALA content of soybean oil through classic plant-breeding techniques and, more recently, by genetic manipulation. This is attractive to the edible-oil industry for use in a nonhydrogenated liquid salad oil and in deep-frying applications because of the increased oxidative stability. Low-ALA soybean varieties with much lower ALA contents, in the range of 3–4%, than the common *williams* variety (ie, ≈7.8%) are now becoming commercially available (13).

The ALA contents of plants vary by season and region. In western Canada, from which the United States gets most of its canola oil, the ALA content of canola oil ranged from 9.0% to 11.8% in the period of 1982–1996 ($\bar{x}$: 10.8%; 14). Regionally, the reported ALA contents of canola oil from Alberta, Manitoba, and Saskatchewan were 10.3%, 9.9%, and 9.4%, respectively (15). The average ALA content of flaxseed oil from western Canada in 1996 was 58.7%. There is some seasonal variation, however, in ALA content. For example, the average ALA content of flaxseed oil ranged from 52% in 1989 to 59% in 1993 (16). The ALA content in western Canadian flaxseed oil also appears to vary markedly from region to region in reports showing that ALA contents of flaxseed oil from Manitoba, Saskatchewan, and Alberta were 57.8%, 59.3%, and 69.8%, respectively (15). As with other highly unsaturated vegetable oils, the ALA content of

TABLE 3
Terrestrial plant sources of α-linolenic acid (18:3n−3)[1]

Source (100-g edible portion, raw)	α-Linolenic acid
	g
Nuts and seeds	
Almonds	0.4
Beechnuts (dried)	1.7
Butternuts (dried)	8.7
Chia seeds (dried)	3.9
Flaxseed	22.8
Hickory nuts (dried)	1.0
Mixed nuts	0.2
Peanuts	0.003
Pecans	0.7
Soybean kernels (roasted or toasted)	1.5
Walnuts, black	3.3
Walnuts, English and Persian	6.8
Vegetables	
Beans, navy, sprouted (cooked)	0.3
Beans, pinto, sprouted (cooked)	0.3
Broccoli (raw)	0.1
Cauliflower (raw)	0.1
Kale (raw)	0.2
Leeks (freeze-dried)	0.7
Lettuce, butterhead	0.1
Lettuce, red leaf	0.1
Mustard	0.1
Purslane[2]	0.4
Radish seeds, sprouted (raw)	0.7
Seaweed, Spirulina (dried)	0.8
Soybeans, green (raw)	3.2
Soybeans, mature seeds, sprouted (cooked)	2.1
Spinach (raw)	0.1
Legumes	
Beans, common (dry)	0.6
Chickpeas (dry)	0.1
Cowpeas (dry)	0.3
Lentils (dry)	0.1
Lima beans (dry)	0.2
Peas, garden (dry)	0.2
Soybeans (dry)	1.6
Grains	
Barley, bran	0.3
Corn, germ	0.3
Oats, germ	1.4
Rice, bran	0.2
Wheat, bran	0.2
Wheat, germ	0.7
Wheat, hard red Winter	0.1
Fruit	
Avocados, California (raw)	0.1
Raspberries (raw)	0.1
Strawberries (raw)	0.1

[1] Data from references 5 and 6.
[2] Also contains trace quantities of eicosapentaenoic acid.

TABLE 4
Percentage contribution of individual fatty acids to the total fatty acid intake by US Department of Agriculture Nationwide Food Consumption Survey food categories in the diets of men and women ≥20 y of age[1]

	4:0–10:0		12:0		14:0		16:0		18:0		18:1		18:2n−6		18:3n−3		18:4n−3 + 20:4n−6		20:5n−3 + 22:6n−3	
Food category	M	W	M	W	M	W	M	W	M	W	M	W	M	W	M	W	M	W	M	W
Milk and milk products	42	59	44	45	43	45	18	19	18	19	10	11	2	2	11	12	0.3	0.3	0.1	0.1
Meat, poultry, fish, and mixtures	9	7	10	8	27	22	41	37	40	36	39	36	23	21	29	26	48	39	88	90
Eggs	1	1	1	0.4	1	1	4	2	5	3	4	4	4	3	4	2	34	32	8	6
Dry beans, peas, legumes, nuts, and seeds	0.1	0.1	1	1	1	1	3	3	3	2	3	4	5	5	2	2	6	6	0	0
Grain products	18	19	33	34	16	18	18	19	19	21	21	22	26	26	20	21	11	12	3	4
Fruit	0.1	0.1	1	1	0.1	1	0.4	1	0.1	0.1	0.3	0.4	1	1	1	1	0	0	0	0
Vegetables	3	4	2	3	3	4	7	7	7	7	11	10	18	16	16	16	1	1	0.2	0.4
Fats, oils, and salad dressings	9	10	7	7	8	9	9	10	8	9	10	12	22	25	18	20	0	0	0	0
Sugars, sweets, and beverages	1	1	2	2	1	1	1	1	2	2	1	1	1	1	1	1	0.2	0	0	0

[1] Adapted from reference 1.

flaxseed oil is directly correlated with the linoleic acid content and inversely correlated with the oleic acid content (16).

Animal production practices, particularly the nutrient composition of the diet, can change the fatty acid profile of meat, milk, and eggs. For example, in muscle and adipose tissues of wild and domestic pigs, linoleic acid comprised 32% and 10% of total fatty acids, respectively; arachidonic acid comprised 8.5% and 0.4%, respectively (17). Likewise, the ratio of n−6 to n−3 fatty acids in egg yolk was 1.3:1 from range-fed chickens and 1.9:1 from commercially raised chickens (18).

MANIPULATION OF n−3 FATTY ACIDS IN ANIMAL PRODUCTS

On the basis of what is known about the effect of diet on the amount of n−3 fatty acids in animal products, researchers are manipulating animal feed in an attempt to increase the n−3 content of eggs, milk, and meat. Animal feed enriched with algae, fishmeal, or fish oil correspondingly increases EPA and DHA concentrations in tissues (eg, muscle and egg yolk). Accordingly, feeding animals diets rich in flaxseed or flax oil, which are good sources of ALA, results in increased amounts of ALA in eggs, milk, pork, chicken, and beef. Major obstacles to this innovative technology include the tendency of these fatty acids to oxidize, producing "off" flavors in food products, as well as the added expense of enriching animal feed with n−3 sources. Increasing the α-tocopherol content of a hen's diet when feeding it n−3 fatty acids helps control oxidation and off flavors in eggs and meat (19), but increases the cost of feeding the animals.

Of the animal products enriched with n−3 fatty acids, eggs are currently the only products available on the market. Eggs were probably targeted first because a large percentage of the n−3 content of the hen's diet is transferred to the egg yolk. The Flax Council of Canada notes that one n−3 fatty acid–enriched egg has about the same amount of n−3 fatty acids as 85 g (3 oz) fish (20). However, commercial production of n−3 fatty acid–enriched meat will not proceed until the issues of oxidation, cost, and extent of biohydration of n−3 fatty acids by ruminants (eg, cattle and sheep) are addressed. The extent to which n−3 fatty acids will be incorporated into meat will also depend on the amounts fed to animals raised for meat and the rate of lipid deposition in meat, which affects the amounts of both intermuscular (seam fat) and intramuscular (marbling) fat. Milk enriched with n−3 fatty acids is high in fat, an undesirable trait given consumer trends in buying lower-fat milk. Therefore, the best opportunity for n−3 fatty acid–enriched milk to enter the marketplace may be in the development and production of butter and cheeses high in n−3 fatty acids.

CHANGES IN THE RATIO OF n−6 TO n−3 FATTY ACIDS OVER TIME

Over the course of evolution there appears, on the basis of estimates from studies of Paleolithic nutrition and modern-day diet assessment, to have been a remarkable change in the fat content and fatty acid profile of the human diet (21, 22). The Paleolithic (400 000–45 000 y ago) diet was likely much lower in total fat (≈21% of energy) and saturated fat (7–8% of energy) than our present-day diet (21, 22). Moreover, the diet of our hunter-gatherer ancestors contained approximately the same quantities of n−6 and n−3 fatty acids (ie, the ratio is thought to have been 1:1). Sources of n−6 and n−3 fatty acids were wild plants, animals, and fish (23, 24). Plant seeds are good sources of n−6 fatty acids and the green leaves of wild plants are good sources of ALA. The wild animals and birds that ate these food sources were sources of these fatty acids in the human food chain. Whereas EPA accounted for 4% of fatty acids in the fat of wild animals (18), domestic animals raised for meat production had undetectable amounts of EPA in their tissues.

At the onset of the industrial revolution (≈140 y ago) there was a marked shift in the ratio of n−6 to n−3 fatty acids in the diet; n−6 fatty acid consumption increased at the expense of that of n−3 fatty acids (25). This change reflected the advent of the modern vegetable oil industry as well as the increased use of cereal grains for domestic livestock. Raper et al (7)

TABLE 5
Ratio of n−6 to n−3 fatty acids in the US food supply

Source and time period	n−6:n−3
Raper et al, 1992 (7)	
1935–1939	8.4
1947–1949	9.0
1957–1959	10.2
1967–1969	9.9
1977–1979	10.1
1985	10.3
Authors' assessment	
1985	12.4
1994	10.6

reported a ratio of n−6 to n−3 fatty acids of 8.4:1 between 1935 and 1939 (estimated by annual per capita food use). From 1935 to 1985, this ratio increased to 10.3 (≈23% increase) (**Table 5**). Accompanying these changes has been a shift in the amounts of fats, oils, fruit, vegetables, nuts, coffee, tea, cocoa, and spices consumed. In 1985, these foods accounted for 68% of the ALA content in the food supply. This reflects an increase from the values reported from the periods of 1967–1969 and 1935–1939 (56% and 54%, respectively). To gain a perspective on whether the ratio of n−6 to n−3 fatty acids changed since 1985, we evaluated annual per capita food disappearance data from the US Department of Agriculture (26). As discussed by Ernst (27), food disappearance data are notoriously difficult to use as estimates of intake, especially of fats and oils. Some factors that make this estimation difficult include frying oil that is discarded after use, the extent to which external fat is trimmed from meat cuts, and use of oils for purposes other than eating. Nonetheless, these data are useful in assessing the diet as complimentary measures to other methods used to quantify fat intake. As shown in Table 5, the ratio of n−6 to n−3 fatty acids we calculated for 1985 was slightly higher (12.4:1) than that reported by Raper et al (10.3:1) (7). Note that the ratio declined from 12.4:1 to 10.6:1 between 1985 and 1994. The change in the ratio reflects a greater disappearance of n−6 fatty acids, by ≈5%, and an accompanying increase in the disappearance of n−3 fatty acids, by 20% (**Table 6**). This shift is due largely to changes in vegetable oil consumption patterns and, in particular, a marked increase in the use (ie, disappearance) of canola oil (of ≈5.5 fold), an oil that has an n−6-to-n−3 fatty acid ratio of 2.2:1, which is distinctly different from the other oils presented in Table 6.

Historically, soybean oil has been and remains the predominant vegetable oil in the American diet. On an absolute basis, soybean oil consumption, reflecting both oil and partially hydrogenated fat, with an approximate ratio of 1:1 (28), remained constant between 1985 and 1994 (19 kg per capita) (Table 5). The relative disappearance of soybean oil, however, declined from 81.7% of the total vegetable oil consumed in 1986 to 76.4% in 1996 (**Table 7**). This change reflects the shifting market shares gained or lost by the other vegetable oils. Vegetable oil consumption increased from 23.1 to 25 kg/person from 1985 to 1994 (Table 6). Four oils (ie, soybean, cottonseed, corn, and canola) accounted for 96% of the total vegetable oil use in the United States in 1996. On the basis of these data, n−6 fatty acid intake from these oils increased from 8.4 to 8.8 $kg \cdot person^{-1} \cdot y^{-1}$, and n−3 fatty acid intake availability rose from 0.545 to 0.669 $kg \cdot person^{-1} \cdot y^{-1}$ (Table 6). These changes in vegetable oil consumption are important contributors to the overall change in the ratio of n−6 to n−3 fatty acid in the US diet. This ratio could be reduced further by substituting oils that are high in n−3 fatty acids for those that are high in n−6 fatty acids. Increased canola oil intake, in particular, will decrease the n−6 to n−3 fatty acid ratio of the diet. Of the oils commonly used, canola oil and soybean oil could be substituted for corn oil and cottonseed oil by individuals to decrease the ratio of n−6 to n−3 fatty acids in the diet.

Although these calculations are insightful, they are particularly important because they reveal that the ratio of n−6 to n−3 fatty acids has been relatively stable for the past 40 y and considerably higher than what some believe to be the optimal ratio (ie, 2.3:1). Accordingly, it is obvious that our present diet has not changed sufficiently to meet this recommended ratio. It also is apparent that increasing EPA and DHA from 0.1 to 0.65 g/d is not going to alter the ratio appreciably. The key question that remains is, To what extent can we realistically lower the n−6 to n−3 fatty acid ratio, and how can this be achieved? A ratio of 2.3:1 translates to 6.7 g n−6 fatty acids and 2.9 g n−3 fatty acids in a 8360 kJ (2000 kcal) diet. The difficulty in meeting the recommended ratio is that many foods typically consumed in the American diet simply have a ratio of n−6 to n−3 fatty acids far above 2.3:1. Even if fish consumption is increased to achieve the goal of 0.65 g/d of EPA and DHA, the ratio will not be markedly lowered unless n−6 fatty acid consumption is decreased markedly. On the basis of these estimates, to achieve n–3 fatty acid recommendations in terms of grams and the ratio of n–6 to n–3 fatty acids, the emphasis should be on increasing EPA and DHA and decreasing n–6 fatty acids in the diet.

TABLE 6
Per capita disappearance of dietary n−6 and n−3 fatty acids in 1985 and 1994[1]

	Disappearance		n−6		n−3	
Food	1985	1994	1985	1994	1985	1994
	kg		*g*		*g*	
Beef	33.2	29.0	51.6	45.2	7.4	5
Pork	21.9	22.7	150.9	156.2	4.9	5
Chicken	20.6	28.9	128.4	179.8	11.5	16
Fish	6.8	6.8	24.6	24.6	45.7	45.7
Eggs	14.9	13.9	170.0	158.2	8.9	8.3
Milk						
Whole	49.1	34.5	43.8	32.1	26.0	19.2
2% fat	33.6	34.0	18.8	18.9	11.1	11.2
1% fat	7.3	9.4	1.7	2.1	1.1	1.4
Skim	6.8	13.1	—	—	—	
Cheese	10.7	12.2	63.5	77.1	40.8	45.4
Oils	23.2	25.1	—	—	—	—
Soybean[2]	18.9	19.1	4500.0	4540.0	387.0	391.0
Corn	2.0	1.8	1160.0	1054.0	—	—
Canola	0.3	1.8	65.0	370.0	30.0	169.0
Cottonseed	1.0	1.4	515.0	726.0	2.2	3.1
Animal fat[3]						
Lard	5.9	5.3	482.0	430.0	16.0	14.0

[1] Data from reference 26. Ratio of n−6 to n−3 fatty acids in 1985, 12.4; in 1994, 10.6.

[2] Calculations were based on the presumption that one-half of soybean oil disappearance is as partially hydrogenated fat and that hydrogenated soybean oil contained 12.5% 18:2n−6 and 1.5% 18:3n−3.

[3] Butterfat is included in the whole milk category.

TABLE 7
Disappearance of vegetable oils (in million kilograms) in the United States in 1986 and 1996[1]

Oil	1986	1996
	% (Gg)	
Soybean	81.7 (4924)	76.4 (6159)
Cottonseed	4.3 (260)	5.7 (461)
Olive	0.9 (54)	1.3 (106)
Corn	8.6 (520)	7.2 (584)
Canola	1.4 (82)	7.2 (581)
Sunflower	1.4 (85)	0.8 (68)
Safflower	0.4 (26)	0.3 (21)
Peanut	1.2 (73)	1.0 (84)

[1] As a percentage of vegetable oil consumed. Total consumption in parentheses. From reference 26.

There appear to have been significant changes in the ratio of n−6 to n−3 fatty acids during human evolution. Since the mid-1800s, the ratio has stabilized for the most part with small fluctuations being noted resulting from changes in vegetable oil consumption. Therefore, it is reasonable to speculate that it is possible to reduce the ratio further, but certainly it is unlikely that, on a population basis, we will ever consume a diet similar to that of our ancestors during the Paleolithic period.

MEETING DIETARY RECOMMENDATIONS FOR n−3 FATTY ACIDS

To date, no official dietary recommendations have been made for n−3 fatty acids in the United States. Recommendations for total PUFA intake, however, have been made: 1–2% of energy from linoleic acid is required to prevent a fatty acid deficiency (29) and total PUFA intake should remain at 7% of energy (30) and not exceed 10% of energy (31). Although no formal recommendation for n−3 fatty acid intake has been made in the United States, a group of nutrition scientists has recently provided guidelines for specific recommendations for ALA, EPA, and DHA (**Table 8, Figure 1**; 21). This group suggests that intake of ALA be 2.2 g/d and that of EPA and DHA combined be 0.65 g/d. In addition, this group recommends an upper limit of 6.7 g linoleic acid/d.

Although the United States has not established official dietary recommendations for n−3 fatty acid intake, Canada (32) and the United Kingdom (33) have. Canada recommends a total n−3 fatty acid intake of 1.2–1.6 g/d, which is similar to the recommendation made by nutrition scientists in the United States but does not distinguish between individual n−3 fatty acids. The United Kingdom does distinguish between n−3 fatty acids and recommends that 1% of energy be from ALA and 0.5% be from EPA and DHA combined. The Committee on Medical Aspects of Food Policy, which includes the United Kingdom, recommends that the combined intake of EPA and DHA be 0.2 g/d (34). Australia has recommended that there be moderate increases in sources of n−3 fatty acids from plant foods (ALA) and fish (EPA and DHA) (35). Lastly, the North Atlantic Treaty Organization Advance Workshop on n−3 and n−6 Fatty Acids recommended that the combined intake of EPA and DHA be 0.27% of energy or 0.8 g/d (33).

Interestingly, some recommendations have been made on the basis of the ratio of n−6 to n−3 fatty acids (**Figure 2**). For example, the World Health Organization has recommended a ratio of n−6 to n−3 fatty acids of 5–10:1 (37). Sweden has recommended that this ratio be 5:1 (38), and Japan (39) has recently changed its recommendation from 4:1 to 2:1 (W Lands, personal communication, 1998).

The recommended ratio of n−6 to n−3 fatty acids is 2.3:1 and has been made to maximize the conversion of ALA to DHA (40). Because of competition between n−6 and n−3 fatty acids for desaturase and elongase enzymes, the quantity of linoleic acid in the diet can affect the extent to which ALA is converted to EPA and DHA in vivo. Kinetic studies conducted in vivo (41) have shown that ≈15% of dietary ALA is converted to the long chain n−3 fatty acids [which include 5 fatty acids of which 3 predominate: 20:5, 22:5, and 22:6 at typical intakes of both linoleic acid (15 g/d; 5% of energy) and ALA (2 g/d; 0.6% of energy)]. Quantitatively, this conversion results in ≈300 mg of n−3 long-chain fatty acids being derived via conversion from ALA. When dietary linoleic acid is increased to 30 g/d, conversion of ALA to the long-chain n−3 fatty acids is reduced by ≈40% (41). Thus, the conditions that favor maximal conversion of ALA to EPA and DHA are critically dependent on the amount of linoleic acid in the diet.

The mean ratio of n−6 to n−3 fatty acid intake in the United States is ≈9.8:1 (Figure 2) which is much higher than that recommended (2.3:1). Sixty percent of the population consumes a ratio of 8–12:1 (Figure 2). Hunter (42) has estimated the ratio to be 10 to 11:1, whereas other estimates indicate that, at least for some individuals, it may be as high as 20–25:1 (20). Thus, to achieve the recommended ratio, it is evident that the US diet will need to be modified (discussed below).

With respect to the modifications in the US diet that will be required, one of the more obvious changes will be to increase EPA and DHA intake, from 0.1–0.2 to 0.65 g/d (Table 6). This represents an increase in EPA and DHA intake of >4-fold and a decrease in linoleic acid from 11–16 g/d to 6.7 g/d (upper limit in an 8.4-MJ/d diet).

Dietary recommendations for n−3 fatty acids have been made on the bases of both absolute mass/d (ie, gram quantities) and

TABLE 8
Approximate quantity of fish or vegetable oils high in n−3 fatty acids needed to meet current recommendations

	Current US recommendations[1]		Canadian recommendation
	ALA, 2.2 g/d	EPA + DHA, 0.65 g/d	n−3 Fatty acids, 1.2–1.6 g/d
		g/d	
Fish			
Halibut		46–62	100–131
Mackerel		20–28	45–60
Herring		26–34	57–74
Salmon		42–56	90–117
Tuna		60–80	130–170
Shrimp		170–228	371–485
Oils			
Canola	24.2		14–18
Menhaden		2.6–3.4	5–7
Soybean	32.2		19–25
Walnut	21.9		12–16

[1] From reference 21.

relative to n−6 fatty acid intake (ie, the ratio of n−6 to n−3 fatty acids). Variables that affect the ratio are energy intake, total PUFA intake and the absolute quantities of n−6 and n−3 fatty acids in the diet. As is apparent, depending on how the recommendation is made for n−3 fatty acid intake (ie, mass per day, percentage of energy, or the ratio of n−6 to n−3 fatty acids per day) different quantities of the n−3 fatty acids would need to be consumed to meet the recommendations.

Expressed on the basis of energy intake (ie, British Nutrition Foundation recommendation), the intakes of EPA + DHA and ALA would need to be increased by 10 and almost 2 times, respectively, to meet the dietary recommendation. Expressed simply as a recommended ratio of 2.3:1, the intake of total n−3 fatty acids would need to be increased by 1.4 and 3.6 g/d if PUFA intake was 4% or 7% of energy intake, respectively, within a 9200-kJ (≈2200 kcal) diet. Thus, it is apparent that depending on the specific dietary recommendation for n−3 fatty acids, the absolute quantity required in the diet can vary appreciably. Because of this inconsistency, a uniform dietary recommendation for n−3 fatty acids is clearly needed. Expressing the recommendation on a mass basis or as a percentage of energy would enable specific dietary recommendations to be made for ALA and for EPA and DHA combined.

It is evident from this discussion that there are several different plausible scenarios in which quantitative and qualitative recommendations for n−3 fatty acid intake can be met. First, the quantity of n−3 fatty acids could meet current recommendations (in grams) yet the ratio of n−6 to n−3 fatty acids could be considerably higher than 2.3:1. This would occur at high energy intakes as well as at high intakes of n−6 fatty acids. For example, if 14 630 kJ (3500 kcal) were consumed in a diet that provided 7% of energy from PUFAs, of which n−3 fatty acids comprised the upper level recommended (ie, 2.87 g/d), the n−6 to n−3 fatty acid ratio would be 9.4:1. Even at lower energy intakes, [ie, 6685 kJ/d (1600 kcal/d)], if total PUFA intake was relatively high (ie, 10% of energy), and the n−3 fatty acid intake recommendation (2.85 g/d) was met, the n−6 to n−3 ratio would be 5.2:1 which is significantly greater than that recommended. Lastly, the ratio of n−6 to n−3 fatty acids could be met while qualitative intake of the n−3 fatty acids fell short of recommended intake. From this discussion, it is apparent that as energy and PUFA intake increase, even if n−3 fatty acid intake recommendations are met (on a mass basis), additional n−3 fatty acids will need to be added to the diet to achieve the recommended ratio of n−6 to n−3 fatty acids.

A critical issue that must be addressed is how to effectively implement the dietary recommendations for n−3 fatty acids. Specifically, the recommendations must be translated into food choices that allow for the target nutrient goals to be achieved. With respect to n−3 fatty acids, there are 2 major food sources in the diet. Some vegetable oils are rich sources of ALA, and certain fish are rich sources of EPA and DHA.

There are 2 ways that current dietary recommendations for n−3 fatty acids can be translated into food choices. The first approach considers the current recommendation to be guided simply by the quantity of ALA and EPA and DHA recommended irrespective of energy intake, fat intake, or the ratios of dietary saturated (SFAs), monounsaturated (MUFAs), and PUFAs. The second approach considers the energy and total fat content of the diet, as well as the distribution of SFA, MUFA, and PUFA and the ratio of n−6 to n−3 fatty acids. However, this second approach does not specifically distinguish a recommendation for ALA from one for EPA + DHA, as does the current recommendation.

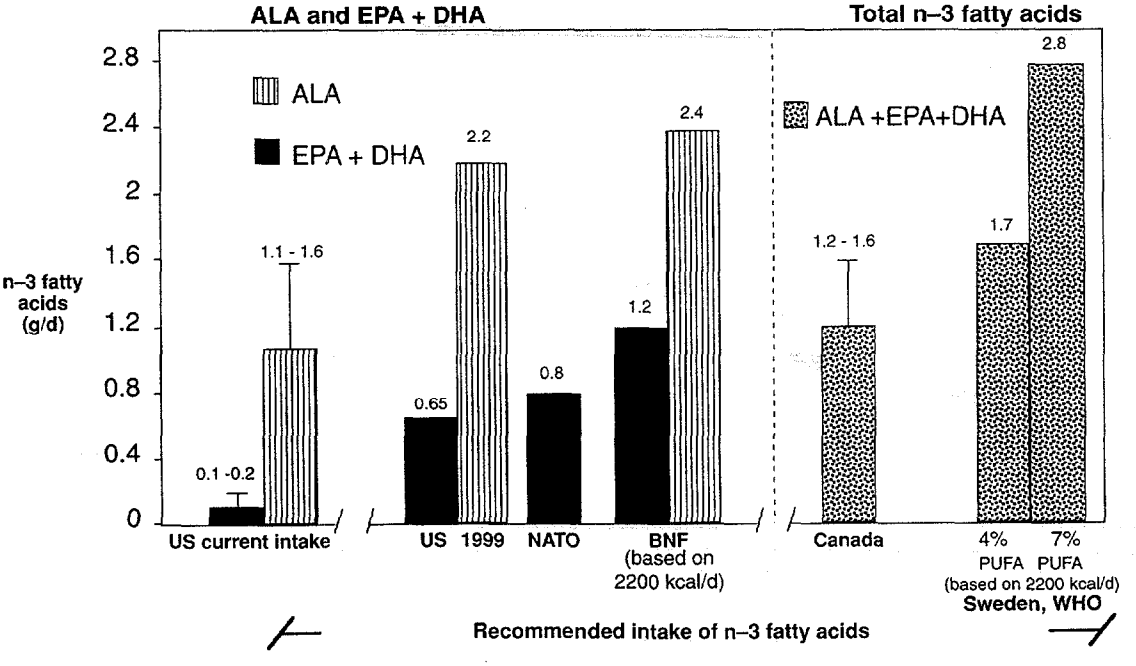

FIGURE 1. Current intake and recommended intakes of α-linolenic acid (ALA) and eicosapentaenoic acid (EPA) plus docosahexaenoic acid (DHA) expressed on the basis of mass/d (current; 21), percentage of energy (British Nutrition Foundation; BNF; 33), and as a ratio of n−6 to n−3 fatty acids of 5:1. The Canadian recommendations (32) are expressed on a mass basis. Gram quantities of EPA + DHA and ALA were calculated on the basis of a 9200-kJ (2200 kcal) diet [BNF, Swedish (38) and World Health Organization (WHO; 33) recommendations] and 4% and 7% of energy from PUFA (Swedish and WHO recommendations). PUFA, polyunsaturated fatty acid.

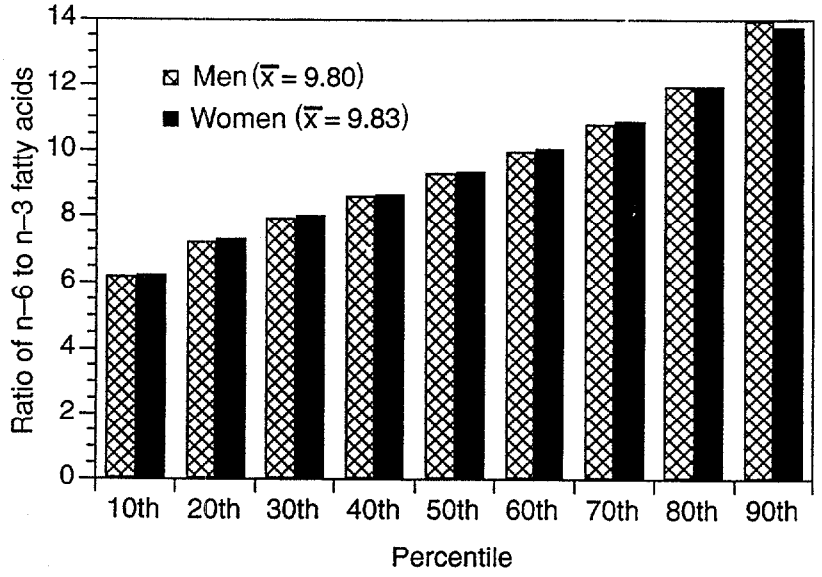

FIGURE 2. Ratio of n−6 to n−3 fatty acids in the diet of men and women in the United States. Data from reference 36.

It is important to recognize that these approaches result in somewhat different quantities of foods (eg, fish and n−3 fatty acid–rich vegetable oils) needed to meet the n−3 fatty acid target goals.

For the current recommendation, ≈20–62 g fatty fish (eg, halibut, mackerel, herring, and salmon) are required per day to meet the EPA and DHA recommendation of 0.65 g/d. Appreciably more lean fish would be required to meet this recommendation. In addition, ≈22–32 g/d of the vegetable oils listed in Table 7 are required to meet the recommendation for ALA. With respect to the Canadian recommendation, which does not distinguish ALA from EPA and DHA, and thus, reflects total n−3 fatty acid intake, it is apparent that if fish were used exclusively to achieve the n−3 fatty acid recommendation, appreciably more fatty fish would have to be included in the diet daily (ie, 45–131 g/d). Likewise, to meet the Canadian recommendation by using vegetable oil exclusively, more oil would need to be included in the diet daily (eg, 12–25 g/d). Thus, specific recommendations for EPA and DHA and for ALA have an appreciable influence on the quantity of fish required, as well as a greater but more subtle effect on the quantity of oil needed in the diet to achieve the recommendation for dietary n−3 fatty acids.

The n−3 fatty acid target can be achieved by including ≈4 fatty fish meals in the diet weekly along with ≈22–32 g/d of a vegetable oil relatively rich in ALA. Use of both fatty fish and oils high in n−3 fatty acids will facilitate the planning of diets that provide recommended amounts of both ALA as well as EPA and DHA. An obvious question that arises is whether the world fish supply is adequate to meet this projected need. Estimates from scientists at the National Fisheries Institute (Roy Martin, personal communication, 1997) indicate that this might be feasible if aquaculture expands rapidly. A 3-fold increase in fish consumption in the United States alone is not attainable because ≈60% of fish eaten in the United States are imported and many stocks are depleted (but recovering). Nonetheless, because aquaculture is one of the fastest growing sectors of agriculture, it is possible that this objective can be met in the long run.

FOOD ENRICHMENT WITH n−3 FATTY ACIDS

Enriching available foods in EPA and DHA provides another option for increasing consumption of these fatty acids. Both oils and powders (produced by microencapsulation technology) enriched with either EPA or DHA are available for infant nutrition specifically. The powdered products can be used in bakery products, milk powders, and salad dressings (43). Technological advances in oil refining have enabled fish oil to be incorporated into vegetable oils for use in the preparation of a wide variety of food products, including canned fish (ie, salmon and tuna). However, foods enriched with high amounts of EPA and DHA sometimes impart a fishy aroma or flavor. Because these food products contain HUFAs that are susceptible to oxidation, considerable efforts have been made to make these products more oxidatively stable during processing, cooking, and storage. Controlling oxidation, and thus, the fishy aroma and flavor, remains a major hurdle in this food enrichment program.

Interestingly, 1 g fish oil provides ≈300 mg EPA + DHA, indicating that this food enrichment program could facilitate our meeting the current recommendations without the need to consume very large quantities of certain foods. Although this is a promising and emerging technology to increase EPA + DHA in the US diet, it is evident that an adequate fish oil supply must be available to meet the projected n−3 fatty acid intake recommendation worldwide.

USE OF BIOTECHNOLOGY TO MODIFY THE HUFA CONTENT OF FOODS

The modern era of biotechnology has witnessed the development of many impressive molecular biology techniques. The advent of these technologies makes it possible to introduce candidate genes that regulate the production of proteins, carbohydrates, or lipids into many plant species of agronomic interest (44, 45). This approach could compliment classical genetic selection programs currently used to modify the nutrient composition of seeds and seed oils. Because of the vast chemical diversity of

plants, the genes required for the synthesis of many different types of lipids exist in nondomesticated species. Although higher plants do not synthesize EPA and DHA, the prospects of introducing the genes that would achieve this is clearly feasible (45). To accomplish this, genes would need to be introduced that elongate 18:4 (found in plants that accumulate γ-linolenic acid) and further desaturate the resulting 20- and 22-carbon fatty acids (45).

The ability to modify the storage lipid composition, and specifically the HUFA content, of commercially important crop plants by transferring the appropriate gene or genes to the desired host plant species is exciting because this biotechnology provides a powerful approach to produce transgenic plants that synthesize "designer" oils. It is not unreasonable to speculate that transgenic soybeans, rape (source of canola oil), or corn will be used to commercially produce oils that are high in EPA and DHA in the future (45). This biotechnology will have a significant effect on human nutrition. The availability of vegetable oils rich in HUFAs will likely play a significant role in helping the US population achieve the EPA + DHA intake goal of 0.65 g/d and, as a result, confer the health benefits ascribed to these unique fatty acids.

SUMMARY

The information presented herein shows clearly that with respect to n−3 fatty acids. EPA and DHA intakes are significantly below amounts that have been recommended by different countries and agencies, as well as by nutritionists in the United States. At the present time, increasing fish consumption by 4-fold is one strategy that will facilitate meeting the recommendations that have been made for intake of EPA and DHA. Manipulating animal feed and enriching food with EPA and DHA are other available options for increasing the intake of these fatty acids. However, it will be necessary to become more reliant on aquaculture to meet the goal of 0.65 g EPA + DHA/d owing to an inadequate supply of fish and fish oil. As further progress is made in producing designer oils via biotechnology, this will offer a complimentary means of increasing EPA and DHA consumption as well as dietary ALA.

Dietary recommendations for n−3 fatty acids have also been made in terms of percentage of energy, as well as on the basis of the ratio of dietary n−6 to n−3 fatty acids. Unlike the recommendation that is expressed on a mass basis, in which a fixed amount is recommended, the amount of n−3 fatty acids recommended using these approaches can vary appreciably among individuals depending on their intakes of energy, total fat, and n−6 fatty acids. Although favorable changes in the ratio of n−6 to n−3 fatty acids can be achieved more easily by decreasing total fat and n−6 fatty acid intakes, this does not necessarily mean that the recommended dose of EPA and DHA is met.

The foregoing summary points to the pressing need in the field to establish dietary recommendations for n−3 fatty acids that distinguish ALA from EPA and DHA. It would be preferable that the recommendation be made on a mass basis (g/d) and not just as a ratio of n–6 to n–3 fatty acids.

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REFERENCES

1. Jonnalagadda SS, Egan SK, Heimbach JT, Harris SS, Kris-Etherton PM. Fatty acid consumption patterns of Americans: 1987–1988 USDA Nationwide Food Consumption Survey. Nutr Res 1995;15:1767–81.
2. Allison DB, Egan SK, Barraj LM, Caughman C, Infante M, Heimbach JT. Estimated intakes of *trans* fatty and other fatty acids in the US population. J Am Diet Assoc 1999;99:166–74.
3. Hepburn FN, Exler J, Weilharach JL. Provisional tables on the content of omega-3 fatty acids and other fat components of selected foods. J Am Diet Assoc 1986;86:788–93.
4. Ackman RG. Concerns for utilization of marine lipids and oils. Food Technol 1988;42:151–5.
5. US Department of Agriculture, Agricultural Research Service, Nutrient Data Laboratory. USDA nutrient database for standard reference, release 12. Nutrient Data Laboratory home page. Version current 1998. World Wide Web: http://www.nal.usda.gov/fnic/foodcomp/ (accessed 3 November 1999).
6. Simopoulos AP. Terrestrial sources of n−3 fatty acids: purslane. In: Quebedeaux B, Bliss F, eds. Horticulture and human health: contributions of fruits and vegetables. Englewood Cliffs, NJ: Prentice-Hall, 1988:93–107.
7. Raper NR, Cronin FJ, Exler J. n−3 Fatty acid content of the US food supply. J Am Coll Nutr 1992;11:304–8.
8. Simopoulos AP, Norman HA, Gillaspy JE, Duke JA. Common purslane: a source of n−3 fatty acids and antioxidants. J Am Coll Nutr 1992;11:374–82.
9. Basiotis PP, Welsh SO, Cronin FJ, Kelsay JL, Mertz W. Number of days of food intake records required to estimate individual and group nutrient intakes with defined confidence. J Nutr 1987;117:1638–41.
10. Dolecek TA, Grandits G. Dietary polyunsaturated fatty acids and mortality in the Multiple Risk Factor Intervention Trial (MRFIT). World Rev Nutr Diet 1991;66:205–16.
11. Aggelousis G, Lazos ES. Fatty acid composition of the lipids from eight freshwater fish species from Greece. J Food Compos Anal 1991;4:68–76.
12. Pigott GM, Tucker BW. Science opens new horizons for marine lipids in human nutrition. Food Rev Int 1987;3:105–38.
13. Perkins FG. Composition of soybeans and soybean products. In: Erikson DR, ed. Practical handbook of soybean processing and utilization. Champaign, IL: AOSC Press and United Soybean Board, 1995:22–3.
14. Grain Research Laboratory, Canadian Grain Commission. Harvest survey of western Canadian canola crop. Crop bulletins. Winnipeg, Canada: Canadian Grain Commission, 1982–1996.
15. Grain Research Laboratory, Canadian Grain Commission. Harvest survey of Western Canadian canola crop. Crop Bulletin no. 230. Winnipeg, Canada: Canadian Grain Commission, 1996.
16. Grain Research Laboratory, Canadian Grain Commission. Quality of western Canadian flaxseed 1996. Crop Bulletin no. 231. Winnipeg, Canada: Canadian Grain Commission, 1996.
17. Crawford MA, Gale MM, Woodford MH. Muscle and adipose tissue lipids of the warthog. *Phacochoerus aethiopicus*. Int J Biochem 1970;1:654–8.
18. Simopoulos AP, Salem N Jr. n−3 Fatty acids in eggs from range-fed Greek chickens. N Engl J Med 1989;321:1412–5.
19. Cherian G, Wolfe FW, Sim JS. Dietary oils with added tocopherols: effects on egg or tissue tocopherols, fatty acids, and oxidative stability. Poult Sci 1996;75:423–31.
20. Sims JS, Qi GH. Designing poultry products using flaxseed. In: Cunnane SC, Thompson LU, eds. Flaxseed in human nutrition. Champaign, IL: AOCS Press, 1995;315–33.
21. Simopolous AP, Leaf A, Salem N Jr. Essentiality of and recommended dietary intakes for omega-6 and omega-3 fatty acids. Ann Nutr Metab 1999;43:127–130.
22. Eaton SB, Konner M. Paleolithic nutrition: a consideration of its nature and current implications. N Engl J Med 1985;312:283–9.
23. Simopoulos AP. n−3 Fatty acids in growth and development and in health and disease. Part 1: the role of n−3 fatty acids in growth and development. Nutr Today 1988;23:10–9.
24. Simopoulos AP. n−3 Fatty acids in growth and development and in health and disease. Part 2: the role of n−3 fatty acids in health and disease: dietary implications. Nutr Today 1988;23:12–8.

25. Leaf A, Weber PC. Cardiovascular effects of n−3 fatty acids. N Engl J Med 1988;318:549–57.
26. Putnam JJ, Allshouse JE. Food consumption, prices, and expenditures, 1970–97. Washington, DC: Food and Rural Economics Division, Economic Research Service, US Department of Agriculture, 1999 (Statistical Bulletin no. 965.)
27. Ernst ND. Fatty acid composition of present day diets. In: Nelson GJ, ed. Health effects of dietary fatty acids. Champaign, IL: AOCS Press, 1991:1–11.
28. Huth PJ. Nutritional aspects of soybean oil and protein. In: Erickson DR, ed. Practical handbook of soybean processing. Champaign, IL: AOCS Press and the United Soybean Board, 1995:460–82.
29. Holman RT. Biological activities of and requirements for polyunsaturated acids. In: Progress in the chemistry of fats and other lipids. New York: Pergamon Press, 1970;607–82.
30. National Research Council. Diet and health. Implications for reducing chronic disease risk. Washington, DC: National Academy Press, 1989.
31. National Cholesterol Education Program. Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. Adult Treatment Panel II. Second report of the Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. Bethesda, MD: The National Heart, Lung, and Blood Institute of the National Institutes of Health, 1993: 3093–5.
32. Scientific Review Committee. Nutrition recommendations. Ottawa: Minister of National Health and Welfare Canada, 1990:H49-42/1990E.
33. British Nutrition Foundation. Unsaturated fatty acids nutritional and physiological significance: the report of the British Nutrition Foundation's task force. New York: Chapman & Hall, 1992.
34. COMA Cardiovascular Review Group. Report on health and social subjects. nutritional aspects of cardiovascular disease. London: Department of Health, 1994:46.
35. NHMRC Working Party Report. The role of polyunsaturated fats in the Australian diet. Melbourne: Kay Gibbons, 1992.
36. Kris-Etherton PM, Taylor DS, Yu S, et al. Present estimates of n−3 fatty acid intakes in the U.S. World Rev Nutr Diet. 1998;83:228 (abstr).
37. World Health Organization. Fats and oils in human nutrition: report of a joint expert consultation. Food and Agriculture Organization of the United Nations and the World Health Organization. FAO Food Nutr Pap 1995:57:1–147.
38. Nordic Working Group on Diet and Nutrition within Nordic Committee of Senior Officials for Food Issues, the Nordic Council of Ministers. Nordic nutrition recommendations 1996. Scand J Nutr 1996;40:161–5.
39. President Summary 1997. J Lipid Nutr 1997;6:5–87.
40. Masters C. n−3 Fatty acids and the peroxisome. Mol Cell Biochem 1996;165:83–93.
41. Emken EA, Adlof RO, Gulley RM. Dietary linoleic acid influences desaturation and acylation of deuterium-labeled linoleic and ALAs in young adult males. Biochim Biophys Acta 1994;1213:277–88.
42. Hunter JE. n−3 Fatty acids from vegetable oils. Am J Clin Nutr 1990;51:809–14.
43. Newton I. Food enrichment with long-chain n−3 PUFA. Food Technol 1996;7:169–77.
44. Hills MJ, Murphy DJ. Biotechnology of oilseeds. Biotechnol Genet Eng Rev 1991;9:41–6.
45. Somerville CR. Future prospects for genetic modification of the composition of edible oils from higher plants. Am J Clin Nutr 1993;58(suppl):270S–5S.

EXHIBIT 6

Fish oil and cardiovascular disease: lipids and arterial function[1,2]

Paul J Nestel

ABSTRACT n−3 Fatty acids have been shown to modify several key risk factors for cardiovascular disease. However, it is not clear whether the apparent protection against cardiovascular disease is directly related to antiatherogenic functions of these fatty acids or is mediated through their modification of the risk factors through mechanisms not directly related to lipids. A major question concerns the importance of lipid modification, which is a potent outcome of fish-oil supplementation. On balance, lipid modification is likely to represent a significant antiatherogenic factor. The benefits include increased HDL_2-cholesterol concentrations, reduced triacylglycerol-rich lipoprotein concentrations, reduced postprandial lipemia, and reduced remnant concentrations. In contrast, LDL-cholesterol concentrations have often been noted to rise and the potential of increased oxidizability of LDLs is potentially adverse with lipid modification, but this potential can be overcome with vitamin E supplementation. The characteristic lipid changes and the underlying mechanisms are reviewed. Additional benefits of fish oils include improved endothelial function and better arterial compliance (elasticity). Future trials will be needed to determine minimum effective dosages of eicosapentaenoic and docosahexaenoic acids over lengthy periods and to show cardiovascular disease reduction through intervention. *Am J Clin Nutr* 2000;71(suppl):228S–31S.

KEY WORDS Fish oil, n−3 fatty acids, eicosapentaenoic acid, docosahexaenoic acid, lipid modification, cardiovascular disease, coronary heart disease, atherogenesis, HDL, LDL-

INTRODUCTION

The active molecules of fish-oil n–3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and possibly other minor fatty acids, are multipotent compounds. Throughout 20 y of research, their potential to counter atherosclerotic vascular diseases has been supported by an increasingly lengthy list of functions, some related to lipid metabolism but others mediated through nonlipid mechanisms. On the negative side, there may be one or more adverse effects of n−3 fatty acids. On balance, if n−3 fatty acids are to be the major explanation for the protection afforded by eating fish, it will be necessary to resolve some key issues. *1)* Which of the effects of EPA, DHA, or both best explain the presumptive protective effects? *2)* Are the changes in lipid metabolism sufficient to provide protection, given that the amounts of n−3 fatty acids needed to show beneficial effects on lipids are far greater than are consumed by fish eaters (other than in unusual populations)? *3)* Could small amounts of n−3 fatty acids be adequate nevertheless if eaten over long periods of time? These issues are shown in **Table 1**.

INFLUENCE OF FISH OILS ON LIPID METABOLISM

Triacylglycerol-rich lipoproteins

The concentrations of endogenously derived triacylglycerol-rich lipoproteins, VLDLs, and intermediate-density lipoproteins have been almost uniformly reported to be lowered with fish oil. Fish oils have been effective in normal subjects and in patients with common phenotypes of hyperlipidemia in which VLDL concentrations are raised. The minimum effective dose of n−3 fatty acids appears to be slightly more than 1 g/d. At intakes >2 g/d, VLDLs decreased an average of 25% in normal subjects and even more in hypertriglyceridemic subjects (≈50% in those with the type 4 or 5 phenotype and ≈40% in those with combined hyperlipoproteinemia) (1). Furthermore, this response is maintained. What of chylomicrons and chylomicron remnants? In more severe forms of hypertriglyceridemia, such as type 5 hyperlipoproteinemia, in which both VLDLs and chylomicrons (or remnants) are present, excess n−3 fatty acids can be highly effective. Whether this result reflects enhanced removal of chylomicrons is uncertain. Catabolized VLDLs and chylomicrons compete for similar removal mechanisms; diminished chylomicron removal may therefore occur whenever VLDL overproduction increases the need for VLDL removal, as in type 5 hyperlipoproteinemia. Chylomicronemia after a fatty meal is diminished when fish oil is eaten over 2 wk (2) but not after a single meal. Remnants in type 3 hyperlipoproteinemia are partly cleared with fish-oil treatment (3).

Dietary fish oils also modify the type of hypertriglyceridemia that is normally inducible by carbohydrates (4). This modification might be expected from the known effects of these 2 nutrients on triacylglycerols: carbohydrates stimulate and fish oils inhibit VLDL production. This is seen strikingly in hepatocytes from obese hyperlipidemic rats in which the usual overproduction of lipid is suppressed with DHA (5).

The nature of the predominant n−3 fatty acids (EPA and DHA) does not seem important in determining plasma triacylglycerol lowering in humans. Fish or fish oils rich in EPA appear to be as effective in humans as is fish rich in DHA. Fish oils vary

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TABLE 1
Treating hyperlipidemia with fish oil: key questions

1) If fish oil inhibits atherosclerosis, how much of this effect is attributable to lipoprotein changes?
2) Is lowering triacylglycerol beneficial?
3) When and why is LDL cholesterol raised and is this necessarily adverse?
4) Is lipoprotein oxidation a threat?
5) Are there benefits secondary to lipid lowering?
6) Given that the above effects require fish oil in amounts exceeding those derived from eating fish, what are the minimal protective amounts of n−3 fatty acids?

considerably in their content of EPA and DHA as well as that of long-chain monoenes and docosapentaenoic acid. A dose-response trial comparing EPA and DHA is urgently needed.

Much larger amounts of fish oil than of individual n−3 fatty acids must be taken to produce an effect. However, whole fish oils are rich in saturated fatty acids that may be undesirable. Therefore, esters of individual n−3 fatty acids have been used. The absorption of the ethyl or methyl esters of EPA appears to be inferior to that of EPA in the glycerides of the fish oils, yet the esters have been therapeutically effective in dosages roughly equal to those in fish oil.

In summary, fish oils affect VLDL metabolism by *1*) reducing VLDL triacylglycerol secretion, as clearly shown in kinetic studies in humans, animal liver perfusions, and isolated hepatocytes (6); *2*) generally, but not always, increasing VLDL apoliprotein B secretion (6, 7) [at least in rat liver, this may be related to increased apolipoprotein B degradation (8), thus assembly of VLDL is impaired]; *3*) reducing triacylglycerol transport, resulting in smaller VLDLs, which are largely converted to LDLs; and *4*) less certainly, increasing VLDL clearance. The key enzyme lipoprotein lipase has mostly been found to be unaffected by fish oil in humans (9).

Chylomicron metabolism

Although there is agreement that chylomicron assembly and secretion are reduced in isolated intestinal cells incubated with EPA, the interpretations of results differ. The mechanisms appear to include the reduction of apolipoprotion B formation and the diversion of EPA from triacylglycerols to phospholipids (10).

Hepatic triacylglycerol metabolism

Reduced triacylglycerol formation is ascribed largely to reduced fatty acid availability. Studies have confirmed that *1*) fish oil increases oxidation of fatty acids by peroxisomal as well as mitochondrial routes (11), which may be mediated by peroxisome proliferator in the liver (12); *2*) fish oil reduces fatty acid synthesis (owing to suppression of key enzymes); *3*) fish oil diverts fatty acids to phospholipids (6); *4*) although fish oil reduces plasma fatty acids, this may be due to increased hepatic uptake through a transporter protein (12); *5*) within the liver, triacylglycerol assembly is impaired through down-regulation of esterifying enzymes (13).

Cholesterol metabolism

Fish oil reduces cholesterol absorption in humans (6) and in monkeys (14). Cholesterol synthesis in the liver is reduced and cholesterol secretion within VLDLs is lowered (6).

LDL metabolism and oxidation

The effects of fish oil on LDL metabolism represent the more controversial aspects of the n−3 fatty acid effects. Why does fish oil cause LDL-cholesterol concentrations to sometimes rise, at least in humans, when all the evidence suggests it should not? Fish oil depresses cholesterol synthesis and may reduce cholesterol absorption (6). This focuses attention on LDL removal and particularly on the LDL (apolipoprotein B/E) receptor. There is evidence that fish oil down-regulates the receptor in hepatic cells (15, 16). Abnormal LDL binding to the receptor in human monocytes (16) and to skin fibroblasts has been ascribed to abnormalities in the LDL itself (17).

Changes in the LDL particles are minor, but tend toward larger, cholesterol-enriched LDLs (18, 19). LDL size relates to the exchange of lipids between LDL, VLDL, and HDL. Fish oil would reduce such exchanges through suppression of cholesterol ester transfer protein and thus favor larger LDL particles (18). Reduced LDL synthesis has been reported with large amounts of fish oil (20).

The n−3 enrichment renders LDLs susceptible to oxidation, as has been shown in several reported studies, with some exceptions. The obvious relevance is to atherogenesis, which is favored by oxidized lipoproteins. The evidence includes increases in in vitro copper-oxidized and macrophage-modified changes in LDL that lead to their increased uptake by macrophages (19). These findings define a potential atherogenic property of dietary fish oil, although it must be emphasised that these are in vitro observations and that the sum of the metabolic outcomes of marine n−3 fatty acids appears to be antiatherogenic in life. Nevertheless, our findings indicate a need for increased antioxidant action, such as that provided by α-tocopherol, if large amounts of fish oil are to be consumed. We have in fact shown that, at least in vitro, the addition of vitamins E and C to n−3 fatty acid–enriched macrophages inhibits their capacity to oxidize LDLs (21). In a study in pigs fed atherogenic diets, however, atherosclerosis was not increased in animals fed fish oil, despite evidence of raised in vitro LDL oxidizability (22).

HDL metabolism

Most reports indicate a favorable effect of fish oil on HDLs. The number of larger cholesterol-rich HDLs (in the HDL_2 range) increases at the expense of HDL_3 (18). However, very high intake of fish oil may lower HDL concentrations (6).

The major effect of fish oil on HDL metabolism is mediated by a reduction in activity of cholesterol ester transfer protein (18), which transfers cholesterol esters from HDLs to VLDLs and LDLs, largely in exchange for VLDL triacylglycerols. Because triacylglycerol concentrations are also reduced, exchange is further diminished, favoring large cholesterol-rich HDL (and LDL) particles over the formation of triacylglycerol-enriched HDLs (and LDLs), which are more susceptible to catabolism.

FISH OIL AND ARTERIAL DISEASE

Data supporting a relation between fish oil and arterial disease are summarized in **Figure 1** and only a few will be discussed further. Other aspects are discussed elsewhere in the supplement.

The reduction in triacylglycerols is one of the modifications in the risk profile. High triacylglycerol concentrations are now widely recognized as an independent risk factor for cardiovascular disease, although the coexistence of low HDL or high LDL concentrations augments the risk substantially. The atherogenicity of intermediate-density lipoproteins, the remnant of VLDL catabolism, is being rediscovered (23).

Modification of dyslipidemia has been the most characteristic effect of fish oils. Triacylglycerol-rich lipoproteins are almost invariably reduced by mechanisms that are now mostly understood. Postprandial lipemia is reduced (9) and potentially atherogenic remnants are cleared. This facilitation of triacylglycerol catabolism partly explains the desirable rise in HDL_2-cholesterol concentrations.

The myocardium is certainly protected from the full damage of ischemia in animals fed fish oil, in which the infarct size is smaller, blood flow is better maintained, and several metabolic disturbances (eg, oxidative damage and calcium overload) that can induce arrhythmias are modified. The protection by fish oils of the myocardium, together with reduction of risk factors and the beneficial modification of arterial responses, explain much of the favorable effectiveness of fish oils (24).

Endothelium-dependent dilatation of arteries is enhanced by fish oils, which also inhibit the vasoconstrictive effects of sympathetic overactivity and norepinephrine (25). We showed that the vascular resistance in the microcirculation of the forearm (which mimics that in the coronary circulation) that occurs when norepinephrine or angiotensin II are infused is attenuated by taking fish oil (25). The improvement might have been due in part to the better lipid profile, because dyslipidemia impairs endothelial function. (Blood pressure was not altered in this study, although this risk factor is reducible in hypertensive subjects.) Endothelial dysfunction is now a well-recognized cause of clinical symptoms in cardiovascular disease and its reversal improves prognosis.

Another index of arterial function is compliance, a measure of the elasticity of large arteries, including the thoracic aorta. Compliance has been reported to be improved by treating diabetic patients (in whom compliance is low as arteries stiffen), with fish oil (26). Of importance is that this improvement in function is achieved within a few weeks.

FISH OR FISH OIL?

The underlying support for fish oil in the management of cardiovascular risk is the apparent protection that eating fish provides. Several large studies have documented such protection from relatively small amounts of fish eaten regularly (27–29). However, this was not observed in a large US study, the Health Professionals Follow-up Study, published in 1995 (30). The most plausible explanation for this exceptional finding is that the average consumption of fish (or fish oil) was already high in these individuals, reducing the likelihood of showing a dose-related response. The current consensus is that eating fish is beneficial at surprisingly modest intakes, and the benefit probably depends on the fatty acid profile of the fish consumed.

We reported that when equivalent amounts of n−3 fatty acids (4 g/d) are eaten as fish or as fish oil, the risk reduction may be greater with fish (31). A recent report of Tanzanian villagers showed that eating fish (3–5 g n−3 fatty acids/d) outperformed vegetarianism in risk factor reduction (32).

Because fish oils will likely be prescribed for patients with or at risk of clinical cardiovascular disease, at issue is whether this will be in the form of the whole fish oils or more purified fatty acids. This will depend on results of future research on whether EPA, DHA, or both in conjunction have superior therapeutic characteristics.

FAVORABLE
Epidemiologic evidence
Experimental atherosclerosis
Risk factor reduction
Modification of atherogenic processes
Protection of endothelial function
Protection of myocardial function

UNFAVORABLE
Equivocal intervention results

FIGURE 1. Evidence of a relation between fish oil and arterial disease.

REFERENCES

1. Harris WS. Fish oils and plasma lipid and lipoprotein metabolism in humans. J Lipid Res 1989;30:785–807.
2. Weintraub MS, Zechner R, Brown A, Eisenberg S, Breslow JL. Dietary polyunsaturated fats of the omega-6 and omega-3 series reduce postprandial lipoprotein levels. J Clin Invest 1988;82:1884–93.
3. Dallongeville J, Boulet L, Davignon J, Lussier-Cacan S. Fish oil supplementation reduces beta-very low density lipoprotein in type III dysbetalipoproteinemia. Arterioscler Thromb 1991;11:864–71.
4. Harris WS, Connor WE, Inkeles SB, Illingworth DR. Dietary omega-3 fatty acids prevent carbohydrate-induced hypertriglyceridemia. Metabolism 1984;33:1016–9.
5. Wong S, Reardon M, Nestel PJ. Reduced triglyceride formation from long-chain polyenoic fatty acids in rat hepatocytes. Metabolism 1985;34:900–5.
6. Nestel PJ. Effects of n−3 fatty acids on lipid metabolism. Annu Rev Nutr 1990;10:149–67.
7. Nestel PJ, Connor WE, Reardon MF, Connor S, Wong S, Boston R. Suppression by diets rich in fish oil of very low density lipoprotein production in man. J Clin Invest 1984;74:82–9.
8. Wang H, Chex X, Fisher EA. n−3 Fatty acids stimulate intracellular degradation of apoprotein B in rat hepatocytes. J Clin Invest 1993;91:1380–9.
9. Harris WS, Connor WE, Alam N, Illingworth DR. Reduction of postprandial triglyceridemia in humans by dietary n−3 fatty acids. J Lipid Res 1988;29:1451–60.
10. Murthy S, Albright E, Mathur SN, Field FJ. Modification of CaCo-2 cell membrane fatty acid composition by eicosapentaenoic acid and palmitic acid: effect on cholesterol metabolism. J Lipid Res 1988;29:773–80.
11. Rustan AC, Christiansen EN, Drevon CA. Serum lipids, hepatic glycerolipid metabolism and peroxisomal fatty acid oxidation in rats fed omega-6 and omega-3 fatty acids. Biochem J 1992;283:333–8.
12. Barrans A, Jaspard B, Barbaras R, Chap H, Perret B, Collet X. Pre-βHDL: structure and metabolism. Biochim Biophys Acta 1996; 1300:73–85.
13. Marsh JB, Topping DL, Nestel PJ. Comparative effects of dietary fish oil and carbohydrate on plasma lipids and hepatic activities of phosphatidate phosphohydrolase, diacyglycerol acyltransferase and neutral lipase activities in the rat. Biochim Biophys Acta 1987;922: 239–43.
14. Parks JS, Crouse JR. Reduction of cholesterol absorption by dietary oleinate and fish oil in African green monkeys. J Lipid Res 1992; 33:559–68.
15. Roach PD, Kambouris AM, Trimble RP, Topping DL, Nestel PJ. The effects of dietary fish oil on hepatic high density and low density lipoprotein receptor activities in the rat. FEBS Lett 1987;222: 159–62.

16. Lindsey S, Pronczuk A, Hayes KC. Low density lipoprotein from humans supplemented with n−3 fatty acids depresses both LDL receptor activity and LDLr mRNA abundance in HepG2 cells. J Lipid Res 1992;33:647–58.
17. Linga V, Leight MA, Curtiss LK, Marcel YL, Richard W, et al. Dietary fish oil-induced decrease in low density lipoprotein binding to fibroblasts is mediated by apolipoprotein E. J Lipid Res 1994;35:491–500.
18. Abbey M, Clifton P, Kestin M, Belling B, Nestel PJ. Effect of fish oil on lipoproteins, lecithin:cholesterol acyltransferase, and lipid transfer protein activity in humans. Arteriosclerosis 1990;10: 85–94.
19. Suzukawa M, Abbey M, Howe PRC, Nestel PJ. Effects of fish oil fatty acids on low density lipoprotein size, oxidizability, and uptake by macrophages. J Lipid Res 1995;36:437–84.
20. Illingworth DR, Harris WS, Connor WE. Inhibition of low density lipoprotein synthesis by dietary omega-3 fatty acids in humans. Arteriosclerosis 1984;4:270–5.
21. Suzukawa M, Abbey M, Clifton P, Nestel PJ. Enhanced capacity of n−3 fatty acid–enriched macrophages to oxidize low density lipoprotein mechanisms and effects of antioxidant vitamins. Atherosclerosis 1996;124:157–69.
22. Whitman SC, Fish JR, Rand ML, Rogers KA. n−3 Fatty acid incorporation into LDL particles renders them more susceptible to oxidation in vitro but not necessarily more atherogenic in vivo. Arterioscler Thromb 1994;14:1170–6.
23. Nestel PJ. New lipoprotein profiles and coronary heart disease. Circulation 1990;82:649–51.
24. Nestel PJ. Fish oil and cardiac function. World Rev Nutr Diet 1991; 66:268–77.
25. Chin JPF, Gust AP, Nestel PJ, Dart AM. Marine oils dose-dependently inhibit vasoconstriction of forearm resistance vessels in humans. Hypertension 1993;21:22–8.
26. McVeigh GE, Brennan GM, Cohn JN, et al. Fish oil improves arterial compliance in non-insulin-dependent diabetes mellitus. Arterioscler Thromb 1994;14:1425–9.
27. Kromhout D, Feskens EJ, Bowles CH. The protective effect of small amounts of fish on coronary heart disease mortality in an elderly population. Int J Epidemiol 1995;24:340–5.
28. Daviglus ML, Stamler J, Orencia AJ, et al. Fish consumption and the 30-year risk of fatal myocardial infarction. N Engl J Med 1997; 336:1046–53.
29. Siscovick DS, Ragunathan TE, King I, et al. Dietary intake and cell membrane levels of long-chain n−3 polyunsaturated fatty acids and the risk of primary cardiac arrest. JAMA 1995;274:1363–7.
30. Ascherio A, Rimm EB, Stampfer MN. Dietary intake of marine n−3 fatty acids, fish intake, and the risk of coronary disease among men. N Engl J Med 1995;332:977–82.
31. Cobiac L, Clifton PM, Abbey M, Belling GB, Nestel PJ. Lipid, lipoprotein, and hemostatic effects of fish vs fish-oil n−3 fatty acids in mildly hyperlipidemic males. Am J Clin Nutr 1991;53: 1210–6.
32. Pauletto P, Puato M, Caroli MG, Casiglia E, Munhambo AE. Blood pressure and atherogenic lipoprotein profiles of fish-diet and vegetarian villagers in Tanzania: the Lugalawa study. Lancet 1996;348:784–8.

EXHIBIT 7

Effect of long-chain n−3 polyunsaturated fatty acids on fasting and postprandial triacylglycerol metabolism[1,2]

Helen M Roche and Michael J Gibney

ABSTRACT Elevated plasma triacylglycerol concentrations have been associated with increased risk of coronary heart disease (CHD). In the past, the epidemiologic evidence about the causal role of triacylglycerols in CHD has not been well regarded, but recent prospective evidence shows that nonfasting plasma triacylglycerol concentration is a strong and independent predictor of future myocardial infarction. Elevated plasma triacylglycerol concentrations are associated with other CHD risk factors, namely reduced HDL-cholesterol concentrations and a preponderance of highly atherogenic, small, dense LDL particles. Plasma triacylglycerol concentrations increase after the ingestion of a fat-containing meal, and elevated postprandial triacylglycerolemia leads to a series of metabolic reactions that reduce HDL-cholesterol concentrations and promote the formation of small, dense LDL particles. The magnitude of the postprandial response is largely determined by fasting plasma triacylglycerol concentrations. Metabolism of plasma triacylglycerols also influences postprandial factor VII activation and the postprandial lipemic responsiveness to dietary cholesterol. Therefore, dietary factors that improve fasting plasma triacylglycerol concentrations must have a role in a healthy diet. Eicosapentaenoic and docosahexaenoic acids are n−3 polyunsaturated fatty acids (PUFAs) in fish oil that effectively reduce plasma triacylglycerol concentrations. Because n−3 PUFAs are effective at low doses (1 g n−3 PUFA/d), they provide a realistic option for the optimization of plasma triacylglycerol metabolism. *Am J Clin Nutr* 2000;71 (suppl):232S–7S.

KEY WORDS Triacylglycerol, postprandial lipemia, n−3 polyunsaturated fatty acids, PUFAs, fish oil, eicosapentaenoic acid, EPA, docosahexaenoic acid, DHA, coronary heart disease

INTRODUCTION

Fish oils are rich sources of the long-chain n−3 polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA; 20:5n−3) and docosahexaenoic acid (DHA; 22:6n−3). Epidemiologic evidence has shown that consumption of n−3 PUFAs is inversely associated with incidence of coronary heart disease (CHD; 1, 2). Prospective studies have shown that relatively low doses of n−3 PUFAs reduce the risk of secondary coronary events (3, 4). The biochemical bases of the ameliorative effect of n−3 PUFAs are thought to be inhibition of coagulation (5), promotion of vasodilation (6), attenuation of inflammation (7), and modification of plasma lipid and lipoprotein concentrations (8). The effect of n−3 PUFAs on plasma lipid and lipoprotein metabolism has been reviewed extensively. In a review of 44 intervention studies that supplemented with a range of 0.5 to 25 g n−3 PUFA/d for an average of 6 wk, it was shown that supplementation had little effect on plasma LDL- and HDL-cholesterol concentrations, but that it consistently and significantly reduced plasma triacylglycerol concentrations (9).

Although elevated plasma triacylglycerol concentrations have been associated with increased risk of CHD, the role of triacylglycerol as an independent risk factor has remained unclear. The epidemiologic evidence regarding the role of plasma triacylglycerol concentrations in CHD was reviewed (10), and multivariate statistical analysis showed no consistent relation between plasma triacylglycerol metabolism and CHD in 5 of the 8 prospective epidemiologic studies. Therefore, the NIH Consensus Development Panel on Triglyceride, High-Density Lipoprotein, and Coronary Heart Disease concluded that "For triglyceride, the data are mixed, and although strong associations are found in some studies, the evidence of a causal relationship is still incomplete" (11). However, more recently, a body of evidence has grown that supports the hypotheses that postprandial triacylglycerol metabolism plays a causal role in the pathogenesis and progression of CHD, and that nonfasting plasma triacylglycerol concentrations are a strong and independent predictor of future myocardial infarction. This article reviews both the evidence in relation to triacylglycerol metabolism and CHD and the efficacy of n−3 PUFAs as hypotriacylglycerolemic agents.

IS PLASMA TRIACYLGLYCEROL CONCENTRATION A RISK FACTOR FOR CHD?

The controversial nature of the triacylglycerol-CHD hypothesis is probably a function of the dynamic nature of the lipid and its carrier lipoproteins. Plasma triacylglycerol concentrations are highly variable, both within and between individuals. Furthermore, plasma triacylglycerol metabolism affects the composition

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and metabolic fate of the HDL and LDL fractions. There is a consistent, negative correlation between plasma triacylglycerol and HDL-cholesterol concentrations, which reflects the physiologic relation between both indexes. Several researchers have proposed *1*) that the correlation between plasma triacylglycerol and HDL_2-cholesterol concentrations may explain why triacylglycerol does not emerge as an independent risk factor in epidemiologic studies, and *2*) that lowered HDL-cholesterol concentrations may only be a marker of plasma triacylglycerol metabolism, not an independent risk factor for CHD (12, 13). This hypothesis is supported by results of the Lipid Research Clinics 12-y follow-up study (14), which showed that individuals with low HDL-cholesterol and high plasma triacylglycerol concentrations showed elevated coronary mortality. Retrospective analysis of the Framingham Heart Study data also showed that high plasma triacylglycerol concentration was a significant risk factor when HDL-cholesterol concentrations were also low (15). The Caerphilly and Speedwell Collaborative Heart Disease Studies concluded that high plasma triacylglycerol concentrations associated with low HDL-cholesterol concentrations predicted subsequent ischemic heart disease events (16). A meta-analysis of 12 population-based, prospective studies showed that the relative risk of CHD increased significantly with increasing plasma triacylglycerol concentrations, and when the relation between triacylglycerol and CHD was adjusted for HDL cholesterol, plasma triacylglycerol concentration remained a significant risk factor (17).

In light of the close metabolic relation between plasma triacylglycerol and HDL-cholesterol concentrations, Sprecher et al (18) proposed the "conjoint trait" hypothesis, which purports that the combination of low HDL-cholesterol and high plasma triacylglycerol concentrations represents a single, inherited phenotype. Subsequent studies have extended this hypothesis and have shown that this phenotype is transmitted across generations as a combined phenotype, or conjoint trait. Multivariate statistical genetic analysis of the qualitative variation in plasma lipid concentrations in first degree relatives has shown that 25% of the genetic variance in plasma triacylglycerol and HDL-cholesterol concentrations may be explained by shared genes, whereas the remaining variability may be accounted for by other unshared genes, environmental factors, or both (19).

High plasma triacylglycerol concentrations are also associated with a preponderance of small, dense LDL particles. This highly atherogenic lipoprotein fraction has been associated with an increased risk (4–6-fold) of coronary artery disease (CAD; 20). Plasma triacylglycerols have a major metabolic influence on the physicochemical properties of LDLs (21). A recent prospective study that investigated the association between LDL particle size and CAD showed that LDL particle size was significantly smaller in CAD patients than in control subjects. Furthermore, multiple stepwise regression analysis identified triacylglycerol as the single most important explanatory variable for LDL size ($R^2 = 0.52$) (22).

This extensive evidence shows the central role of plasma triacylglycerol metabolism in determining the composition and metabolic fate of the other lipoproteins (LDL and HDL); therefore, the causal role of plasma triacylglycerol in the pathogenesis of CHD may have been underestimated. This view is supported by prospective evidence that nonfasting triacylglycerol concentration is a strong and independent predictor of future myocardial infarction (23). In this study, increased nonfasting plasma triacylglycerol concentrations were also associated with the presence of small, dense LDL particles and low HDL-cholesterol concentrations.

POSTPRANDIAL TRIACYLGLYCEROL METABOLISM AND CHD

In light of the importance of plasma triacylglycerol concentrations and the variability in plasma triacylglycerols associated with dietary fat intake, the body of research investigating the relation between postprandial triacylglycerol metabolism and CHD is growing. The postprandial triacylglycerolemic response refers to a series of metabolic events that occur after ingestion of a fat-containing meal. This process has been reviewed extensively elsewhere (24, 25), and a brief synopsis is presented here.

Dietary fat is composed principally of triacylglycerol, which, after digestion and absorption (26, 27), stimulates the production of chylomicrons. These triacylglycerol-rich lipoproteins (TRLs) transport dietary triacylglycerol within the circulation, causing an increase in plasma triacylglycerol concentrations. The magnitude of the postprandial response is determined by several factors; it increases with fasting plasma triacylglycerol concentration, age, and sedentary lifestyle and is greater in males than in females. Nutritional factors, including fat dose and habitual dietary fat composition, affect the magnitude of the postprandial lipemic response, which is attenuated markedly by chronic n−3 PUFA intake.

Several clinical studies have shown that postprandial lipemia is an important factor in the pathogenesis and progression of CHD. It has been shown that men with CAD had pronounced and delayed postprandial lipemia compared with control subjects (28). It has also been shown that postprandial, but not fasting, triacylglycerol concentration was the most accurate (68%) predictor of the presence and progression of atherosclerosis (29), even when all the traditionally accepted risk factors of CHD were included in the multivariate regression analysis. Karpe et al (30) investigated the postprandial lipemic response in male postinfarction patients and age-matched control subjects and showed that the concentration of postprandial chylomicron remnant apolipoprotein B48 was directly related to the rate of progression of coronary lesions.

ELEVATED POSTPRANDIAL TRIACYLGLYCEROL CONCENTRATIONS AND LIPOPROTEIN METABOLISM

An increase in plasma triacylglycerol concentrations is a normal metabolic consequence after ingestion of dietary fat. However, elevated postprandial plasma triacylglycerol concentrations are associated with several adverse metabolic events, including the formation of atherogenic chylomicron remnants, the formation of small, dense LDL particles, and the reduction of plasma HDL-cholesterol concentrations (24). Zilversmit (31) was the first to propose that cholesteryl ester–rich chylomicron remnants were as atherogenic as LDLs. Since then it has been shown that an elevated postprandial response is associated with the formation and increased concentrations of small, cholesteryl ester–enriched chylomicron remnants. These remnants share with LDL the ability to mediate cholesterol influx into the arterial wall intima in humans (32), thereby promoting atherogenesis. The close inverse association between HDL cholesterol and plasma triacylglycerol concentrations may be explained by the metabolic effects of elevated postprandial plasma triacylglycerol concentrations. Efficient postprandial lipid metabolism, with rapid clearance of chylomicrons, promotes HDL formation (33, 34). In contrast, excessive

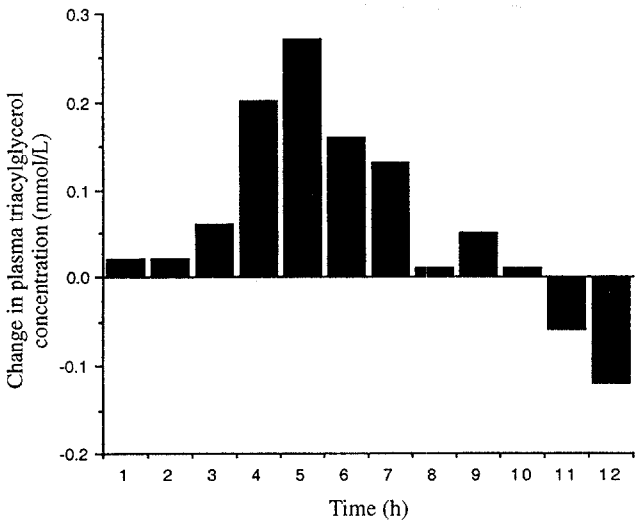

FIGURE 1. Change in plasma triacylglycerol concentration after ingestion of a meal containing 40 g fat. From reference 40.

postprandial triacylglycerol concentrations lead to excessive enrichment of HDL2 with triacylglycerol (35, 36). Hepatic lipase delipidates the triacylglycerol-rich HDL2 particles, converting them to small, dense HDL3 particles, thus lowering the concentration of the cardioprotective and metabolically active HDL2 fraction.

Excessive postprandial triacylglycerol concentrations promote the formation of the highly atherogenic small, dense LDL particles (20). Cholesterol esterification accelerates during postprandial lipemia and cholesteryl-ester transfer protein (CETP) catalyzes the heteroexchange of cholesterol ester from LDL for triacylglycerol from TRLs. These triacylglycerol-enriched particles then become dense LDLs. Karpe et al (37) showed that the magnitude of the postprandial TRL response and lipoprotein lipase (LPL) activity accounted for ≈50% of the variability of the distribution of LDL particles between the light and dense subfractions. A moderately high plasma triacylglycerol concentration, a low HDL-cholesterol concentration, and an increased proportion of LDL as small, dense LDLs comprise the atherogenic lipoprotein phenotype (ALP), which is accepted as the most common dyslipidemia associated with increased risk of CHD (38). Although the metabolic interplay between these lipoproteins provides a plausible explanation for this lipoprotein profile, it is proposed that there may also be a genetic basis for this common phenotype, such as the conjoint trait hypothesis discussed previously.

CRITICISMS OF CURRENT APPROACHES TO THE INVESTIGATION OF POSTPRANDIAL TRIACYLGLYCEROL METABOLISM

Postprandial investigations usually collect a series of postprandial blood samples from subjects who fasted overnight and consumed a single fat-rich test meal. It is important to realize that the requirement to standardize clinical postprandial investigations leads to an artificial situation that may not necessarily reflect the free-living postprandial state. Issues relating to the preprandial fast, the dose of fat, and the timing of fat intake represent key issues that affect the postprandial response but do not necessarily reflect a free-living situation.

Fasting plasma triacylglycerol concentration is the single most influential factor in the magnitude of the postprandial triacylglycerol response. There is a strong positive association between fasting plasma triacylglycerol concentrations and the magnitude of the postprandial triacylglycerolemic response (39); therefore, factors that affect fasting plasma triacylglycerol concentrations are important. The definition of fasting is unclear, but it generally means an overnight or 12-h fast. A typical postprandial triacylglycerolemic response after the ingestion of a meal containing 40 g fat is presented in **Figure 1** (40). Plasma triacylglycerol concentrations peaked 4–5 h after the meal and returned to baseline by 8 h after. As the test progressed, plasma triacylglycerol concentrations fell below baseline values and appeared to decrease linearly with time. Therefore, 12 h after meal ingestion, subjects' triacylglycerol concentrations were lower than the initial, fasting concentrations. There is a need to determine the optimum pretest conditions for postprandial investigations because the pretest diet, physical activity, and the timing and composition of the last meal all influence the preprandial or fasted state and also affect the postprandial response.

A second difficulty in relation to postprandial triacylglycerol studies is the dose of fat used. In the literature this has ranged from 20 to 120 g (41, 42). The mean amount of dietary fat consumed by 38 free-living volunteers consuming self-selected diets at different eating occasions throughout the day is shown in **Figure 2** (43). Note that the mean fat intake at each eating occasion ranged between 12 and 30 g, the fat dose being low in the early morning and higher in the early evening. These data challenge the relevance of postprandial investigations because *1*) the quantities of fat consumed as part of a habitual diet are much lower than those used in postprandial investigations, *2*) individuals consume dietary fat as part of 3–6 eating occasions (44) rather than as 1 bolus, and *3*) most postprandial investigations begin in early morning, a stage in the circadian rhythm when fat intake tends to be low. Whereas it is understandable that high fat doses are used to exaggerate the metabolic sequelae of the postprandial response to understand the biochemical basis of the postprandial triacylglycerolemic response, it cannot be assumed that the observed effects also occur in free-living situations. This hypothesis is to some extent borne out by the Physicians Health Study, in which nonfasting plasma triacylglycerol concentrations strongly and independently predicted subsequent myocardial infarction, and this relation was not influenced by the duration of fast before plasma triacylglycerol was measured (23). Clearly, investigations of postprandial lipid metabolism in the free-living situation are needed.

FASTING PLASMA TRIACYLGLYCEROL AND RISK FACTORS FOR CORONARY HEART DISEASE

Although the continued investigation of postprandial lipemia to elucidate the biochemical basis of plasma TRL metabolism in relation to CHD is important, there is ample evidence that elevated fasting plasma triacylglycerol concentrations as such are indicative of abnormal postprandial lipoprotein metabolism. Therefore, the relation between fasting plasma triacylglycerol concentrations and other CHD risk factors should not be ignored. For example, increased fasting triacylglycerol concentrations are associated with reduced concentrations of HDL cholesterol and, as previously discussed, this results from the remodeling of HDLs during postprandial lipemia that affects the composition and metabolic fate of HDLs. Likewise, 2 recent studies showed that fasting plasma triacylglycerol concentration, a marker for

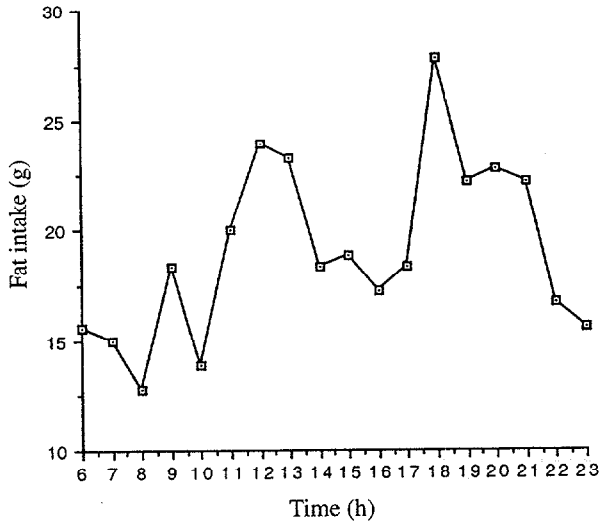

FIGURE 2. Fat intake of free-living volunteers consuming self-selected diets over 18 h. From reference 43.

the postprandial triacylglycerol response, has important effects on other CHD risk factors. The first relates to the activation of factor VII during postprandial lipemia (45). Several investigators have shown that coagulation factor VII is activated during postprandial triacylglycerolemia and that this response is affected by other lifestyle factors, including age (46), habitual dietary fat composition (47, 48), test-meal fat dose (49) and test-meal composition (49, 50). Although these factors play a role in the postprandial response, this effect is minimal compared with the effect of fasting plasma triacylglycerol concentrations (45). The importance of fasting plasma triacylglycerol was shown with multiple regression analysis that identified fasting plasma triacylglycerol concentration as the most important determinant of fasting factor VII activity, which in turn determined the magnitude of the postprandial response. Therefore, fasting plasma triacylglycerol concentrations determine not only the magnitude of the postprandial triacylglycerol response but also that of the postprandial thrombotic response.

Fasting plasma triacylglycerol concentrations also determine the effect of dietary cholesterol. The postprandial response to a low-fat, high-cholesterol test meal was investigated in 3 groups with different lipoprotein phenotypes (normal lipid concentrations, high LDL-cholesterol concentrations, and high plasma triacylglycerol concentrations; 51). This study showed that the group with high fasting plasma triacylglycerol concentration had a greater increase in the concentration of triacylglycerol and cholesterol in the TRL fraction. Considering the strong association between plasma triacylglycerol and cholesterol concentration with CHD, this study provides considerable evidence that fasting plasma triacylglycerol concentration is an important determinant of the effect of dietary cholesterol on postprandial lipemia. These effects of fasting plasma triacylglycerol concentrations on HDL-cholesterol concentration, factor VII activity, and cholesterol absorption are mediated through an abnormal postprandial triacylglycerol metabolism. Even so, fasting plasma triacylglycerol concentration is a marker for this abnormal state; therefore, lifestyle factors—including diet—that alter fasting plasma triacylglycerol deserve intensive research.

LIFESTYLE FACTORS THAT INFLUENCE PLASMA TRIACYLGLYCEROL CONCENTRATIONS

Several physiologic factors affect plasma triacylglycerol concentrations and the magnitude of the postprandial lipemic response. Fasting and postprandial plasma triacylglycerol concentrations are greater in men than in women (52, 53) and tend to increase with age (52, 54). Therefore, fasting plasma triacylglycerol concentrations account, in part, for differences in magnitude of postprandial lipemia associated with age and sex. The lower fasting triacylglycerol concentrations and postprandial lipemic response found in female subjects could be mediated through greater activity of lipoprotein lipase (LPL), the hydrolytic enzyme responsible for the removal of the TRL triacylglycerol from the circulation (55). Women have more adipose tissue LPL than do men (53). LPL activity is also increased after physical exercise conditioning (56), which accounts for reduced postprandial triacylglycerolemia in subjects who participate in physical exercise (56, 57). Obesity is associated with a greater postprandial triacylglycerol response, which may also be related to greater fasting triacylglycerol concentrations (58).

Dietary factors also affects the magnitude of the postprandial lipemic response. n−3 PUFAs are well known hypotriacylglycerolemic agents. Several studies have shown that n−3 PUFAs reduce plasma triacylglycerol concentrations dose dependently (59–61). When the data from these studies were pooled (**Figure 3**), it emerged that the change in fasting plasma triacylglycerol (ΔT) is related to the dose of n−3 PUFA intake (P) according to the equation $\Delta T = -7.67 - 3.05P$ ($R^2 = 0.874$). However, this equation relates to doses of n−3 PUFAs ranging between 1 and 9 g/d. Furthermore, this analysis does not take into account other important factors, ie, initial (preintervention) n−3 PUFA intake and duration of supplementation, which affect the triacylglycerol-lowering capacity of n−3 PUFAs. Lower doses of n−3 PUFAs supplemented over a longer intervention period (16 wk) have been shown to be effective hypotriacylglycerolemic agents. In that study, 1 g n−3 PUFAs/d reduced fasting plasma triacylglycerol concentrations by 21.2%, a level much greater than predicted from the above equation (10.7%) (8). This low dose of

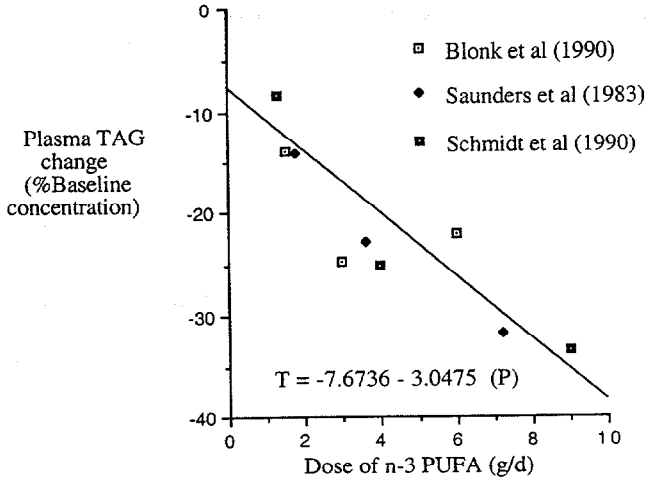

FIGURE 3. The dose-dependent hypotriacylglycerolemic effect of n−3 PUFA supplementation. Data from references 59–61. Reproduced with permission from reference 8.

n−3 PUFAs also reduced postprandial triacylglycerol concentrations significantly, to a degree similar to that seen in studies that used a much higher dose of n−3 PUFAs (24 g/d) (62, 63).

This hypotriacylglycerolemic effect of n−3 PUFAs may be mediated through reduced endogenous TRL synthesis, increased TRL removal, or a combination of both. Diminished endogenous TRL production has been shown in kinetic studies, in which the protein moiety of VLDLs was radiolabeled and n−3 PUFAs reduced VLDL synthesis (64). Chylomicrons and VLDLs compete for LPL-mediated removal from the circulation (65). Therefore, reduced VLDL synthesis would promote chylomicron removal, thus reducing the postprandial triacylglycerolemic response. The presence of n−3 PUFAs in a meal also promotes removal of TRL from the circulation (63), and postheparin LPL activity is significantly greater after a n−3 PUFA meal compared with a saturated fatty acid–rich test meal (66). Rat studies have shown that fish-oil consumption leads to significantly higher expression of adipose tissue LPL messenger RNA (67), which suggests that n−3 PUFA supplementation increases LPL-mediated TRL clearance. It is probable that n−3 PUFAs mediate their effect on plasma triacylglycerol concentrations by both reducing endogenous VLDL production and increasing TRL removal.

CONCLUSION

It is clear that in the future, plasma triacylglycerol concentrations should be considered an important factor in relation to the development of CHD. Although studying the postprandial triacylglycerol response allows investigation of the metabolic sequelae that occur postprandially, the standard protocol whereby subjects consume a high-fat dose in the early morning may not reflect the free-living situation. Fasting plasma triacylglycerol concentrations are reliable markers for the magnitude of the postprandial response. Therefore, this measure should be used to indicate abnormalities of postprandial triacylglycerol metabolism, namely reduced HDL-cholesterol concentration, a preponderance of small, dense LDL particles; excessive factor VII activation; and excessive cholesterol absorption. n−3 PUFAs are effective hypotriacylglycerolemic agents, even when consumed at low doses; therefore, increasing consumption of n−3 PUFAs will improve triacylglycerol metabolism both in the fasted and postprandial state and should be promoted as part of a healthy diet.

REFERENCES

1. Bang HO, Dyerberg J. Plasma lipids and lipoproteins in Greenlandic West-coast Eskimos. Acta Med Scand 1972;192:85–94.
2. Hirai A, Hamazaki T, Terano T, et al. Eicosapentaenoic acid and platelet function in Japanese. Lancet 1980;2:1132–3.
3. Burr ML, Fehily AM, Gilbert JF, et al. Effects of changes in fat, fish, and fibre intakes on death and myocardial reinfarction: diet and reinfarction trial (DART). Lancet 1989;2:757–61.
4. de Lorgeril M, Renaud S, Mamelle N, et al. Mediterranean alpha-linolenic acid-rich diet in secondary prevention of coronary heart disease. Lancet 1994;343:1454–9.
5. Leaf A, Weber PC. Cardiovascular effects of n−3 fatty acids. N Engl J Med 1988;318:549–57.
6. Bruckner G, Webb P, Greenwell L, Chow C, Richardson D. Fish oil increases peripheral capillary blood velocity in humans. Atherosclerosis 1987;66:237–45.
7. Wallace JM, Turley E, Gilmore WS, Strain JJ. Dietary fish oil supplementation alters leukocyte function and cytokine production in healthy women. Arterioscler Thromb Vasc Biol 1995;15:185–9.
8. Roche HM, Gibney MJ. Postprandial triacylglycerolaemia: the effect of low-fat dietary treatment with and without fish oil supplementation. Eur J Clin Nutr 1996;50:617–24.
9. Harris WS. Fish oils and plasma lipid and lipoprotein metabolism in humans: a critical review. J Lipid Res 1989;30:785–807.
10. Austin MA. Plasma triglyceride as a risk factor for coronary heart disease. Am J Epidemiol 1989;129:249–59.
11. NIH Consensus Conference. Triglyceride, high-density lipoprotein, and coronary heart disease. NIH Consensus Development Panel on Triglyceride, High-Density Lipoprotein, and Coronary Heart Disease. JAMA 1993;269:505–10.
12. Brunzell JD, Austin MA. Plasma triglyceride levels and coronary disease. N Engl J Med 1989;320:1273–5.
13. Schwandt P. The triglyceride controversy: a review of the data. Eur Heart J 1990;11:38–43.
14. Criqui MH, Heiss G, Cohn R, et al. Plasma triglyceride level and mortality from coronary heart disease. N Engl J Med 1993;328:1220–5.
15. Castelli WP. The triglyceride issue: a view from Framingham. Am Heart J 1986;112:432–7.
16. Bainton D, Miller NE, Bolton CH, et al. Plasma triglyceride and high density lipoprotein cholesterol as predictors of ischaemic heart disease in British men. The Caerphilly and Speedwell Collaborative Heart Disease Studies. Br Heart J 1992;68:60–6.
17. Gordon DJ, Rifkind BM. High-density lipoprotein—the clinical implications of recent studies. N Engl J Med 1989;321:1311–5.
18. Sprecher DL, Hein MJ, Laskarzewski PM. Conjoint high triglycerides and low HDL cholesterol across generations. Analysis of proband hypertriacylglycerolaemia and lipid/lipoprotein disorders in first-degree family members. Circulation 1994;90:1177–84.
19. Mahaney MC, Blangero J, Comuzzie AG, et al. Plasma HDL cholesterol, triglycerides, and adiposity. A quantitative genetic test of the conjoint trait hypothesis in the San Antonio Family Heart Study. Circulation 1995;92:3240–8.
20. Griffin BA, Packard CJ. Metabolism of VLDL and LDL subclasses. Curr Opin Lipidol 1994;5:200–6.
21. Griffin BA, Freeman DJ, Tait GW, et al. Role of plasma triglyceride in the regulation of plasma low density lipoprotein (LDL) subfractions: relative contribution of small, dense LDL to coronary heart disease risk. Atherosclerosis 1994;106:241–53.
22. Gardner CD, Fortmann SP, Krauss RM. Association of small low-density lipoprotein particles within the incidence of coronary heart disease in men and women. JAMA 1996;276:875–81.
23. Stampfer MJ, Krauss RM, Ma J, et al. A prospective study of triglyceride level, low-density lipoprotein particle diameter, and risk of myocardial infarction. JAMA 1996;276:882–8.
24. Roche HM, Gibney MJ. Postprandial triacylglycerolaemia—nutritional implications. Prog Lipid Res 1995;34:249–66.
25. Sethi S, Gibney MJ, Williams CM. Postprandial lipoprotein metabolism. Nutr Res Rev 1993;6:161–83.
26. Carey MC, Small DM, Bliss CM. Lipid digestion and absorption. Annu Rev Physiol 1983;45:651–77.
27. Small DM. The effects of glyceride structure on absorption and metabolism. Annu Rev Nutr 1991;11:413–34.
28. Groot PHE, van Stiphout WAHJ, Krauss XH, et al. Postprandial lipoprotein metabolism in normolipidemic men with and without coronary artery disease. Arterioscler Thromb 1991;11:653–62.
29. Patsch JR, Miesenbock G, Hopferwieser T, et al. Relation of triglyceride metabolism and coronary artery disease. Arterioscler Thromb 1993;12:1336–45.
30. Karpe F, Steiner G, Uffelman K, Olivecrona T, Hamsten A. Postprandial lipoproteins and progression of coronary atherosclerosis. Atherosclerosis 1994;106:83–97.
31. Zilversmit DB. Atherogenesis: a postprandial phenomenon. Circulation 1979;60:473–85.
32. Shaikh M, Wootton R, Nordestgaard BG, et al. Quantitative studies of transfer in vivo of low density, SF 12-60, and SF 60-400 lipoproteins between plasma and arterial intima in humans. Arterioscler Thromb 1991;11:569–77.

33. Nikkila EA, Taskinen M-R, Sane T. Plasma high-density lipoprotein concentration and subfraction distribution in relation to triglyceride metabolism. Am Heart J 1987;113:543–8.
34. De Bruin TWA, Brouwer CB, Gimpel JA, Erkelens DW. Postprandial decrease in HDL cholesterol and HDL apo A-1 in normal subjects in relation to triglyceride metabolism. Am J Physiol 1991;260:E492–8.
35. Patsch JR, Prasad S, Gotto AM, Bengtsson-Olivecrona G. Postprandial lipemia. A key for the conversion of high density lipoprotein$_2$ into high density lipoprotein$_3$ by hepatic lipase. J Clin Invest 1984;74:2017–23.
36. Patsch JR, Prasad S, Gotto AM, Patsch W. High density lipoprotein$_2$. Relationship of the plasma levels of this lipoprotein species to its composition, to the magnitude of postprandial lipemia, and to the activities of lipoprotein lipase and hepatic lipase. J Clin Invest 1987;80:341–7.
37. Karpe F, Tornvall P, Olivecrona T, Steiner G, Carlson LA, Hamsten A. Composition of human low density lipoprotein: effects of postprandial triglyceride-rich lipoproteins, lipoprotein lipase, hepatic lipase and cholesteryl ester transfer protein. Atherosclerosis 1993; 98:33–49.
38. Austin MA, King MC, Vranizan KM, Krauss RM. Atherogenic lipoprotein phenotype. A proposed genetic marker of coronary heart disease risk. Circulation 1990;82:495–506.
39. O'Meara KM, Lewis GF, Cabana VG, Iverius PH, Getz GS, Polonsky KS. Role of basal triglyceride and high density lipoprotein in determination of postprandial lipid and lipoprotein responses. J Clin Endocrinol Metab 1992;75:465–71.
40. Gibney MJ, Daly E. The incorporation of n−3 polyunsaturated fatty acids into plasma lipid and lipoprotein fractions in the postprandial phase in healthy volunteers. Eur J Clin Nutr 1994;48:866–72.
41. Murphy MC, Isherwood SG, Sethi S, et al. Postprandial lipid and hormone responses to meals of varying fat contents: modulator role of lipoprotein lipase? Eur J Clin Nutr 1995;49:579–88.
42. Cohen JC, Noakes TD, Spinnler Benade AJ. Serum triglyceride responses to fatty meals: effects of meal fat content. Am J Clin Nutr 1988;47:825–7.
43. De Castro JM. Circadian rhythms of the spontaneous meal pattern, macronutrient intake, and mood of humans. Physiol Behav 1987; 40:437–46.
44. McGrath SA, Gibney MJ. The effects of altered frequency of eating on plasma lipids in free-living healthy meals on normal self selected diets. Eur J Clin Nutr 1994:48:402–7.
45. Roche HM, Gibney MJ. Postprandial coagulation factor VII activity: the effect of monounsaturated fatty acids. Br J Nutr 1997;77:537–49.
46. Kario K, Matsuo T. Lipid-related hemostatic abnormalities in the elderly: imbalance between coagulation and fibrinolysis. Atherosclerosis 1993;103:131–8.
47. Miller GJ, Martin JC, Mitropoulos KA, et al. Plasma factor VII is activated by postprandial triglyceridaemia, irrespective of dietary fat composition. Atherosclerosis 1991;86:163–71.
48. Tholstrup T, Marckmann P, Jespersen J, Sandstrom B. Fat high in stearic acid favorably affects blood lipids and factor VII coagulant activity in comparison with fats high in palmitic acid or high in myristic and lauric acids. Am J Clin Nutr 1994;59:371–7.
49. Salomaa V, Rasi V, Pekkanen J, et al. The effects of saturated fat and n−6 polyunsaturated fat on postprandial lipemia and hemostatic activity. Atherosclerosis 1993;103:1–11.
50. Mitropoulos KA, Miller GJ, Martin JC, Reeves BEA, Cooper J. Dietary fat induces changes in factor VII coagulant activity through effects on plasma free stearic acid concentration. Arterioscler Thromb 1994;14:214–22.
51. Clifton PM, Nestel PJ. Effect of dietary cholesterol on postprandial lipoproteins in three phenotypic groups. Am J Clin Nutr 1996;64:361–7.
52. Cohn JS, McNamara JR, Cohn SD, Ordovas JM, Schaefer EJ. Postprandial plasma lipoprotein changes in human subjects of different ages. J Lipid Res 1988;29:469–79.
53. Kashyap, ML, Barnhart, RL, Srivastava, LS, et al. Alimentary lipemia: plasma high-density lipoproteins and apolipoproteins CII and CIII in healthy subjects. Am J Clin Nutr 1983;37:233–43.
54. Krasinski SD, Cohn JS, Schaefer EJ, Russell RM. Postprandial plasma retinyl ester response is greater in older subjects compared with younger subjects. Evidence for delayed plasma clearance of intestinal lipoproteins. J Clin Invest 1990;85:883–92.
55. Eckel RH. Lipoprotein lipase. A multifunctional enzyme relevant to common metabolic diseases. N Engl J Med 1989;320:1060–8.
56. Weintraub MS, Rosen Y, Otto R, Eisenberg S, Breslow JL. Physical exercise conditioning in the absence of weight loss reduces fasting and postprandial triglyceride-rich lipoprotein levels. Circulation 1989;79:1007–14.
57. Cohen JC, Noakes TD, Benade AJ. Postprandial lipemia and chylomicron clearance in athletes and in sedentary men. Am J Clin Nutr 1989;49:443–7.
58. Lewis GF, O'Meara NM, Soltys PA, et al. Postprandial lipoprotein metabolism in normal and obese subjects: comparison after the vitamin A fat-loading test. J Clin Endocrinol Metab 1990;71:1041–50.
59. Sanders TAB, Roshanai F. The influence of different types of omega 3 polyunsaturated fatty acids on blood lipids and platelet function in healthy volunteers. Clin Sci 1983;64:91–9.
60. Blonk MC, Bilo HJG, Nauta JJP, Popp-Snijders C, Mulder C, Donker AJM. Dose-response effects of fish-oil supplementation in healthy volunteers. Am J Clin Nutr 1990;52:120–7.
61. Schmidt EB, Varming K, Ernst E, Madsen P, Dyerberg J. Dose-response studies on the effect of n−3 polyunsaturated fatty acids on lipids and haemostasis. Thromb Haemost 1990;63:1–5.
62. Harris WS, Connor WE, Alam N, Illingworth RD. Reduction of postprandial triglyceridemia in humans by dietary n−3 fatty acids. J Lipid Res 1988;29:1451–60.
63. Weintraub MS, Zechner R, Brown A, Eisenberg S, Breslow JL. Dietary polyunsaturated fats of the W-6 and W-3 series reduce postprandial lipoprotein levels. J Clin Invest 1988;82:1884–93.
64. Harris WS, Connor WE, Illingworth RD, Rothrock DW, Foster DM. Effects of fish oil on VLDL triglyceride kinetics in humans. J Lipid Res 1990;31:1549–58.
65. Goldberg IJ. Lipoprotein lipase and lipolysis: central roles in lipoprotein metabolism and atherogenesis. J Lipid Res 1996;37:693–707.
66. Zampelas A, Murphy M, Morgan LM, Williams CM. Postprandial lipoprotein lipase, insulin and gastric inhibitory polypeptide responses to test meals of different fatty acid composition: comparison of saturated, n−6 and n−3 polyunsaturated fatty acids. Eur J Clin Nutr 1994;48:849–58.
67. Murphy MC, Zampelas A, Puddicombe SM, Furlonger NP, Morgan LM, Williams CM. Pretranslational regulation of the expression of lipoprotien lipase gene by dietary fatty acids in the rat. Br J Nutr 1993;70:727–36.

EXHIBIT 8

Dietary intake of long-chain n−3 polyunsaturated fatty acids and the risk of primary cardiac arrest[1–3]

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ABSTRACT Whether the dietary intake of long-chain n−3 polyunsaturated fatty acids (PUFAs) from seafood reduces the risk of ischemic heart disease remains a source of controversy, in part because studies have yielded inconsistent findings. Results from experimental studies in animals suggest that recent dietary intake of long-chain n−3 PUFAs, compared with saturated and monounsaturated fats, reduces vulnerability to ventricular fibrillation, a life-threatening cardiac arrhythmia that is a major cause of ischemic heart disease mortality. Until recently, whether a similar effect of long-chain n−3 PUFAs from seafood occurred in humans was unknown. We summarize the findings from a population-based case-control study that showed that the dietary intake of long-chain n−3 PUFAs from seafood, measured both directly with a questionnaire and indirectly with a biomarker, is associated with a reduced risk of primary cardiac arrest in humans. The findings also suggest that *1*) compared with no seafood intake, modest dietary intake of long-chain n−3 PUFAs from seafood (equivalent to 1 fatty fish meal/wk) is associated with a reduction in the risk of primary cardiac arrest; *2*) compared with modest intake, higher intakes of these fatty acids are not associated with a further reduction in such risk; and *3*) the reduced risk of primary cardiac arrest may be mediated, at least in part, by the effect of dietary n−3 PUFA intake on cell membrane fatty acid composition. These findings also may help to explain the apparent inconsistencies in earlier studies of long-chain n−3 PUFA intake and ischemic heart disease. *Am J Clin Nutr* 2000;71(suppl):208S–12S.

KEY WORDS n−3 Fatty acids, diet, risk factors, arrythmia, sudden death, cardiac arrest, ischemic heart disease

INTRODUCTION

Whether the dietary intake of long-chain n−3 polyunsaturated fatty acids (PUFAs) from seafood reduces the risk of ischemic heart disease remains a source of controversy, because studies have yielded inconsistent findings (1–12). The differences among cohorts in the range of dietary intake of long-chain n−3 PUFAs might account for the inconsistent findings: compared with no seafood intake, the modest intake of seafood, ie, 1–2 fatty fish meals/wk, is associated with reduced risk of ischemic heart disease mortality, but there is little evidence that higher intake further reduces risk (13). However, the differences in the ischemic heart disease outcomes examined also might account for the inconsistent findings: there is little evidence that intake of these fatty acids reduces nonfatal ischemic heart disease outcomes such as myocardial infarction and angina pectoris (9–12).

Although coronary atherosclerosis is the major determinant of both ischemic heart disease mortality and nonfatal ischemic heart disease, the acute pathophysiologic mechanisms that lead to various ischemic heart disease outcomes differ. For example, ventricular fibrillation, a cardiac arrhythmia that results in out-of-hospital primary cardiac arrest and a major cause of ischemic heart disease mortality, results in part from an increased myocardial vulnerability to life-threatening arrhythmias (14). Studies in experimental animals suggest that recent dietary intake of long-chain n−3 PUFAs, compared with intake of saturated and monounsaturated fatty acids, reduces myocardial vulnerability to ventricular fibrillation, possibly through an effect on myocardial cell membrane composition (15–20).

In this report, the findings of a population-based case-control study of the dietary intake of long-chain n−3 PUFAs from seafood and the risk of primary cardiac arrest among humans (21) are summarized. Because of the wide range of seafood intake in the community, we examined the dose-response relation between dietary intake of long-chain n−3 PUFAs and the risk of primary cardiac arrest. Because we measured both dietary intake and cell membrane concentrations of long-chain n−3 PUFAs, we also explored whether the relation between dietary intake and the risk of primary cardiac arrest might be mediated through alterations in cell membrane fatty acid composition. The findings of this study may help to explain the inconsistent obser-

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vations from prior cohort studies of dietary intake of long-chain n−3 PUFAs and ischemic heart disease.

METHODS

Briefly, we conducted a population-based case-control study in Seattle and King County, WA. We identified all case subjects with primary cardiac arrest, aged 25–74 y, attended by paramedics during 1988–1994 (n = 334). Control subjects were randomly identified from the same defined population, matched by age (within 7 y) and sex (n = 493). We excluded case and control subjects with prior clinically recognized heart disease or other major life-threatening morbidity and those who had taken fish-oil supplements during the prior year. All subjects were married and were residents of King County; their spouses participated in in-home interviews.

To estimate the dietary intake of long-chain n−3 PUFAs from seafood during the prior month, we developed a quantitative food-frequency questionnaire, the seafood intake scale (SIS). The dietary assessment focused on the prior month because cell membrane composition reflects dietary intake over a period of weeks. The SIS included a list of 25 fish and 10 shellfish available in the Pacific Northwest. For each type of seafood consumed, information was collected on the quantity (usual serving size) and frequency (number of servings) of consumption during the prior month. We estimated the overall intake of eicosapentaenoic acid (EPA; 20:5n−3) and docosahexaenoic acid (DHA; 22:6n−3) from seafood by combining the information from the SIS with information on the average EPA and DHA content of each type of seafood and summing the intake across all types of seafood (22–25).

In substudies, we showed both the validity and the reliability of spouse estimates of dietary long-chain n−3 PUFA intake from seafood. Additionally, on the basis of 8 d of food records collected by control subjects, we showed that the estimates of long-chain n−3 PUFA intake were only weakly related to energy intake and the intake of other nutrients such as saturated fat, protein, carbohydrate, fiber, vitamins, and minerals.

Additionally, we assessed the dietary intake of long-chain n−3 PUFAs from seafood indirectly by using a biomarker, the fatty acid composition of red blood cell membranes. Paramedics obtained blood specimens in the field from a subset of case subjects with primary cardiac arrest after essential emergency medical care had been provided and the patient was either clinically stable or resuscitation had proven ineffective. Data from a preliminary study in 18 primates had suggested that cardiac arrest itself alters long-chain n−3 PUFAs in red blood cell membranes only slightly (21). Blood specimens were obtained from control subjects at the time of the in-person interview. The protocol was approved by the University of Washington Human Subjects Review Committee. Laboratory analyses were conducted to estimate red blood cell membrane combined EPA and DHA, expressed as a percentage of the total cell membrane fatty acids.

We used conditional logistic regression analysis to examine the relation of dietary intake and cell membrane concentrations of long-chain n−3 PUFAs with risk of primary cardiac arrest. To explore the data for a nonlinear dose-response relation, we estimated both the linear and the quadratic terms for dietary intake of long-chain n−3 PUFAs in the logistic model. To determine whether the effect of dietary intake of long-chain n−3 PUFAs might be mediated through alterations in cell membrane fatty acid composition, we also examined the effect of dietary intake on the risk of primary cardiac arrest after adjusting for red blood cell membrane concentrations of long-chain n−3 PUFAs and other clinical characteristics. If dietary intake influences risk through cell membrane fatty acid concentrations, we expected any association between dietary intake and primary cardiac arrest to be reduced or eliminated by the inclusion of the cell membrane fatty acid concentrations in the statistical models.

RESULTS

Both the mean dietary intake and red blood cell membrane concentrations of long-chain n−3 PUFAs were lower in case subjects than in control subjects. Mean (±SD) dietary intakes of combined EPA and DHA were 4.3 ± 6.0 and 5.3 ± 5.6 g/mo for case and control subjects, respectively (P = 0.02). Mean (± SD) red blood cell membrane combined EPA and DHA concentration were 4.3 ± 1.1% and 4.9 ± 1.4% of total fatty acids for case and control subjects, respectively (P = 0.002). There was an inverse relation between the dietary intake of long-chain n−3 PUFAs from seafood and the risk of primary cardiac arrest; however, the addition of the quadratic term for dietary intake of long-chain n−3 PUFAs improved the fit of the logistic model with the linear term alone (P = 0.002), a finding consistent with a nonlinear dose-response relation (**Figure 1**).

Compared with no seafood intake, modest intake of n−3 PUFAs (5.5 g/mo, the equivalent of 1 fatty fish meal/wk, was associated with a 50% reduction in the risk of primary cardiac arrest (odds ratio: 0.5; 95% CI: 0.4, 0.8) after adjustment for age, smoking, family history of myocardial infarction or sudden death, saturated fat intake, hypertension, diabetes, weight, height, physical activity level, and education. Further adjustment for other risk factors, including high blood cholesterol concentrations and alcohol and caffeine intake, altered the findings only slightly. There was little evidence that a higher dietary intake of long-chain n−3 PUFAs was associated with a further reduction in the risk of primary cardiac arrest.

There also was an inverse relation between the combined EPA and DHA concentrations of red blood cell membranes and the risk of primary cardiac arrest (**Figure 2**). The addition of a quadratic term did not improve the fit of the model with the linear term for red blood cell membrane long-chain n−3 PUFA concentration (P = 0.80). Compared with a long-chain n−3 PUFA concentration of 3.3% of total fatty acids (the mean value of the lowest quartile), a red blood cell membrane concentration of 5.0% of total fatty acids (the mean of the third quartile) was associated with a 70% reduction in the risk of primary cardiac arrest (odds ratio: 0.3; 95% CI: 0.2, 0.6), after adjustment for other risk factors.

We also explored whether dietary intake of long-chain n−3 PUFAs might influence the risk of primary cardiac arrest by altering cell membrane fatty acid composition. Among the subset of case and control subjects with blood specimens available, dietary intake of long-chain n−3 PUFAs, assessed directly, was inversely related to risk of primary cardiac arrest, after adjustment for other factors (**Figure 3**). However, after further adjustment for differences in the red blood cell membrane fatty acid concentrations, the dietary intake of long-chain n−3 PUFAs was not associated with the risk of primary cardiac arrest: the odds ratios associated with each quartile of dietary intake assessed directly were close to 1.0.

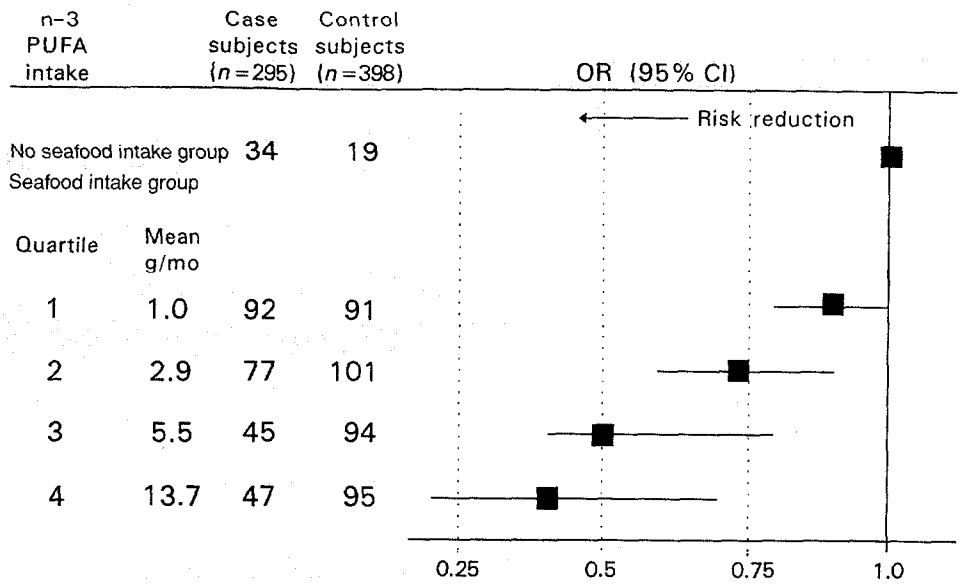

FIGURE 1. Dietary intake of long-chain n−3 polyunsaturated fatty acids (PUFAs) and cardiac arrest. Quartile means are from control subjects. Odds ratios (ORs; ■) and 95% CIs (bars) were from a conditional logistic model that included the linear and quadratic terms for dietary intake of long-chain n−3 PUFAs after adjustment for age, current smoking, former smoking, family history of myocardial infarction or sudden death, fat intake scale (26), hypertension, diabetes mellitus, physical activity level, weight, height, and education. ORs and 95% CIs were calculated by using the no-seafood-intake group as the reference group and the mean value for each seafood intake (long chain n−3 PUFA) category.

DISCUSSION

We assessed the association between the dietary intake of long-chain n−3 PUFAs from seafood and the risk of primary cardiac arrest, an important cause of mortality from ischemic heart disease, in a population with a broad range of dietary intake of long-chain n−3 PUFAs from seafood. Compared with no seafood intake, the consumption of modest amounts of long-chain n−3 PUFAs from seafood [96 g (3 oz) fatty fish/wk, the equivalent of 1 fatty fish meal] was associated with a marked reduction in the risk of primary cardiac arrest. However, there

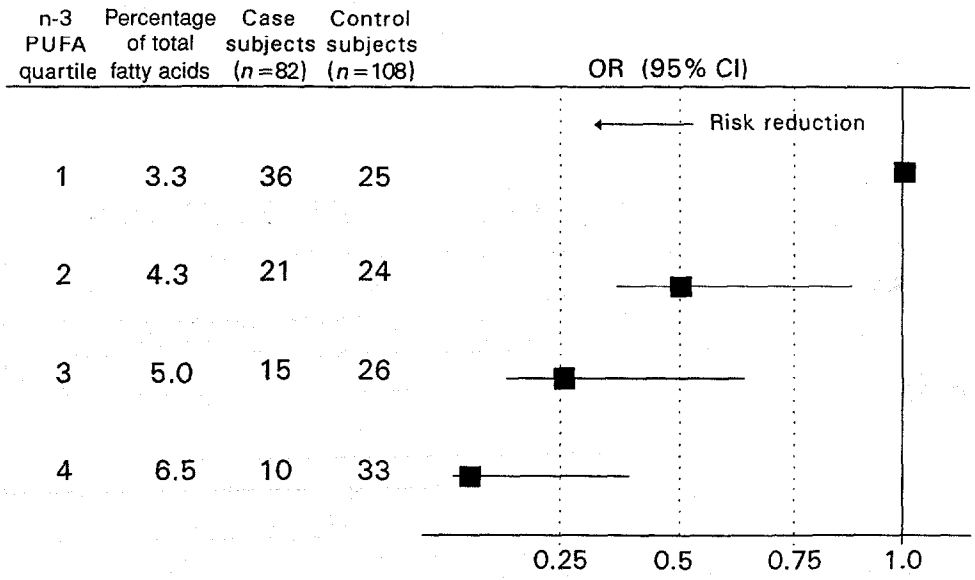

FIGURE 2. Red blood cell membrane long-chain n−3 polyunsaturated fatty acids (PUFAs) and cardiac arrest. Quartile means are from control subjects. Odds ratios (ORs; ■) and 95% CIs (bars) were from a conditional logistic model that included the linear term for red blood cell membrane long-chain n−3 PUFAs after adjustment for age, current smoking, former smoking, family history of myocardial infarction or sudden death, fat intake scale (26), hypertension, diabetes mellitus, physical activity level, weight, height, and education. ORs and 95% CIs were calculated by using the lowest quartile as the reference group and the mean value for each category.

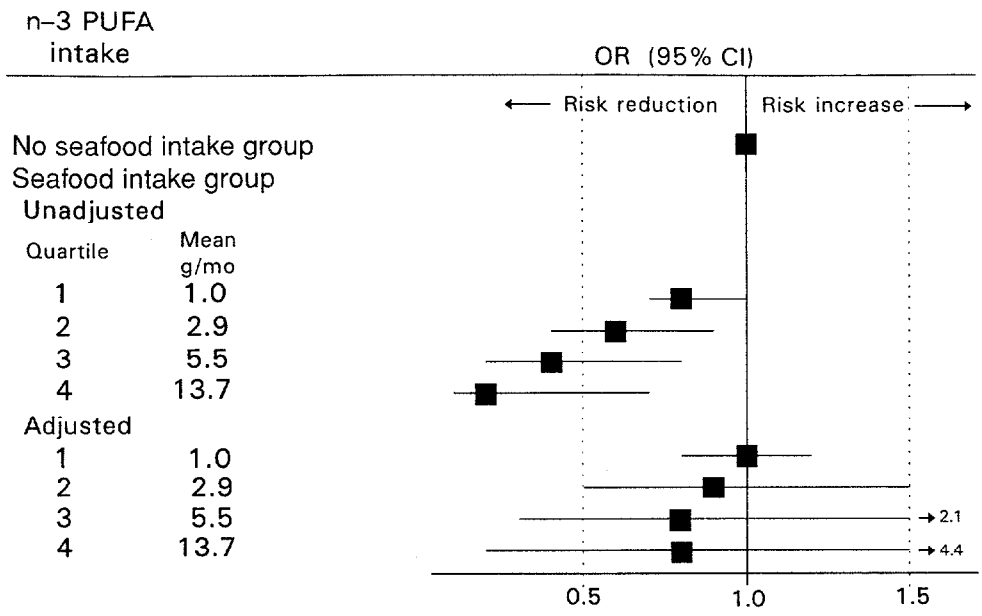

FIGURE 3. Dietary intake of long-chain n−3 polyunsaturated fatty acids (PUFAs) and cardiac arrest with and without adjustment for red blood cell membrane long-chain n−3 PUFAs. Quartile means are from control subjects. Estimates were based on only matched case (n = 82) and control (n = 108) subjects with complete data on red blood cell membrane n−3 fatty acid concentrations and covariates. Odds ratios (ORs; ■) and 95% CIs (bars) are from a conditional logistic model that included the linear and quadratic terms for dietary intake of long-chain n−3 PUFAs, with and without adjustment for red cell membrane long chain n−3 PUFAs, after adjustment for age, current smoking, former smoking, family history of myocardial infarction or sudden death, fat intake scale (26), hypertension, diabetes mellitus, physical activity level, weight, height, and education. ORs and 95% CIs were calculated by using the no-seafood-intake group as the reference group and the mean value of dietary intake for each category.

was little evidence that consumption of higher amounts of long chain n−3 PUFAs was associated with a further reduction in the risk of primary cardiac arrest. Whereas the limitations of an observational study preclude an assessment of potential mechanisms, the findings were consistent with the hypothesis that alterations in cell membrane fatty acid composition may mediate the association between dietary intake of long chain n−3 PUFAs and vulnerability to life-threatening cardiac arrhythmias.

Several limitations of the study need to be considered. We assessed the dietary intake of long-chain n−3 PUFAs by using both a questionnaire and a biomarker: each approach to measurement has limitations. The questionnaire measure relied on the recall of surrogate respondents, ie, the spouses of the subjects. The biomarker measure was influenced also by both intake of other dietary fatty acids and endogenous metabolism of fatty acids. [Of note, the inclusion of docosapentaenoic acid (DPA; 22:5n−3) in the dietary and biomarker estimates of long-chain n−3 PUFA intake from seafood did not alter our findings in a preliminary analysis.] Nevertheless, we suggest that the consistency of the inverse relation between dietary intake of long-chain n−3 PUFAs in 2 different measures that differ in their limitations adds strength to our findings. Also, the results do not preclude bias related to other dietary factors that differ between those who ate seafood and those who did not because we could not obtain a full nutrient assessment from the surrogate respondents. Additionally, the generalizability of our findings to other settings may be limited. The effect of fat intake in the background diet on the observed association is unknown. Furthermore, in other settings, potential adverse effects of toxins found in seafood, eg, mercury, may also alter the benefit-to-risk ratio (12).

Both the magnitude of the risk reduction in primary cardiac arrest and the dose-response relation observed in this study are consistent with the ischemic heart disease mortality risk reduction observed in prior cohort studies (2–12). The findings from this study also are consistent with the findings from 2 secondary prevention trials in patients with a prior myocardial infarction (27, 28). Among men randomly assigned to dietary advice to increase their intake of fish (or n−3 fatty acids from fish oil), there was a 27% reduction in fatal ischemic heart disease but no reduction in the incidence of recurrent nonfatal myocardial infarction (27). In another secondary prevention trial, men randomly assigned to a diet that included a high intake of α-linolenic acid (18:3n−3), the precursor of the long-chain n−3 PUFAs, experienced a significant reduction in total mortality, primarily as a result of a profound reduction in the incidence of cardiac arrest (28). Taken together, these studies suggest that differences in both the range of long-chain n−3 PUFA intake from seafood and the ischemic heart disease outcomes may account for the differences in findings from prior studies.

Studies have also examined the potential mechanisms that underlie these observations (29–31). Studies using isolated myocyte models and voltage clamping have explored the effects of long-chain n−3 PUFAs on the automaticity of isolated cells and the function of sodium, calcium, and potassium channels, respectively. The studies suggest that long-chain n−3 PUFAs may alter electrophysiologic function in a manner that reduces the vulnerability to ventricular fibrillation and that these alterations may explain observations from animal and epidemiologic studies and clinical trials.

For now, public health recommendations to incorporate modest amounts of fatty fish in the diet seem appropriate, given the potential cardiac benefits of modest long-chain n−3 PUFA intake. However, additional primary and secondary prevention trials are needed to evaluate further whether modest dietary intake of long-chain n−3 PUFAs from seafood, low-dose long-chain n−3 PUFA supplements (equivalent in dose to the modest dietary intake from seafood), and intermediate chain n−3 PUFAs, such as α-linolenic acid, reduce ischemic heart disease mortality through a reduction in the incidence of arrhythmic death among persons with low dietary intakes of n−3 PUFAs.

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REFERENCES

1. Stone NJ. Fish consumption, fish oil, lipids, and coronary heart disease. Circulation 1996;94:2337–40.
2. Kromhout D, Bosschieter EB, de Lezenne Coulander C. The inverse relation between fish consumption and 20-year mortality from coronary heart disease. N Engl J Med 1985;312:1205–9.
3. Shekelle RB, MissellL, Paul O, Shryock AM, Stamler J. Fish consumption and mortality from coronary heart disease. N Engl J Med 1985;313:820 (letter).
4. Dolecek TA, Grandits G. Dietary polyunsaturated fatty acids and mortality in the Multiple Risk Factor Intervention Trial (MRFIT). World Rev Nutr Diet 1991;66:205–16.
5. Kromhout D, Feskens EJM, Bowles CH. The protective effect of a small amount of fish on coronary heart disease mortality in an elderly population. Int J Epidemiol 1995;24:340–5.
6. Norell SE, Ahlbom A, Feychting M, Pedersen NL. Fish consumption and mortality from coronary heart disease. Br Med J 1986; 293:426 (letter).
7. Curb JD, Reed DM. Fish consumption and mortality from coronary heart disease. N Engl J Med 1985;313:821 (letter).
8. Vollset SE, Heugh I, Bjelke E. Fish consumption and mortality from coronary heart disease. N Engl J Med 1985;313:820–1 (letter).
9. Morris MC, Manson JE, Rosner B, Buring JE, Willett WC, Hennekens CH. Fish consumption and cardiovascular disease in the Physicians Health Study: a prospective study. Am J Epidemiol 1995;142:166–75.
10. Guallar E, Hennekens CH, Sacks FM, Willett WC, Stampfer MJ. A prospective study of plasma fish oil levels and incidence of myocardial infarction in U.S. male physicians. J Am Coll Cardiol 1995; 25:387–94.
11. Ascherio A, Rimm E, Stampfer MJ, et al. Dietary intake of marine n−3 fatty acids, fish intake, and the risk of coronary heart disease among men. N Engl J Med 1995;332:977–82.
12. Salonen JT, Nyyssonen K, Salonen R. Fish intake and the risk of coronary disease. N Engl J Med 1995;333:937 (letter).
13. Katan MB. Fish and heart disease. N Engl J Med 1995;332:1024–5.
14. Siscovick DS. Primary prevention of primary cardiac arrest. Cardiac Electrophysiol Rev 1997;1:152–4.
15. Charnock JS. Dietary fats and cardiac arrhythmia in primates. Nutrition 1994;10:161–9.
16. McLennan PL, Bridle TM, Abeywardena MY, et al. Dietary lipid modulation of ventricular fibrillation threshold in the marmoset monkey. Am Heart J 1992;123:1555–61.
17. McLennan PL, Abeywardena MY, Charnock JS. The influence of age and dietary fat in an animal model of sudden cardiac death. Aust N Z J Med 1989;19:1–5.
18. Charnock JS, Abeywardena MY, Poletti VM, et al. Differences in fatty acid composition of various tissues of the marmoset monkey after different lipid supplemented diets. Comp Biochem Physiol Comp Physiol 1992;101:387–93.
19. McLennan PL, Bridle TM, Abeywardena MY, Charnock JS. Comparative efficacy of n−3 and n−6 polyunsaturated fatty acids in modulating ventricular fibrillation threshold in marmoset monkeys. Am J Clin Nutr 1993;58:666–9.
20. Billman GE, Hallaq H, Leaf A. Prevention of ischemia-induced ventricular fibrillation by n−3 fatty acids. Proc Natl Acad Sci U S A 1994;91:4427–30.
21. Siscovick DS, Raghunathan TE, King I, et al. Dietary intake and cell membrane levels of long-chain n-3 polyunsaturated fatty acids and the risk of primary cardiac arrest. JAMA 1995;274: 1363–7.
22. Nutrition Monitoring Division, Human Nutrition Information Services. Composition of foods: finfish and shellfish products. Beltsville, MD: US Department of Agriculture, 1987. (Handbook number 8–15.)
23. Nutrition Monitoring Division, Human Nutrition Information Services. Composition of foods: finfish and shellfish products. Belstville, MD: US Department of Agriculture, 1989. (Handbook number 8–15.)
24. Nutrition Monitoring Division, Human Nutrition Information Services. Composition of foods: finfish and shellfish products. Beltsville, MD: US Department of Agriculture, 1990. (Handbook number 8–15.)
25. Nutrition Monitoring Division, Human Nutrition Information Services. Composition of foods: finfish and shellfish products. Beltsville, MD: US Department of Agriculture, 1991. (Handbook number 8–15.)
26. Retzlaff BM, Dowdy AA, Walden CE, Bovbjerg VE, Knopp RH. The Northwest Lipid Research Clinic Fat Intake Scale: validation and utility. Am J Public Health 1997;87:181–5.
27. Burr ML, Fehily AM, Gilbert JF, et al. Effects of changes in fat, fish, and fibre intakes on death and myocardial reinfarction: Diet and Reinfarction Trial (DART). Lancet 1989;334:757–61.
28. Lorgeril MD, Mamelle N, Salen P, et al. Mediterranean alpha-linolenic acid-rich diet in secondary prevention of coronary heart disease. Lancet 1994;343:1454–9.
29. Hallaq H, Smith TW, Leaf A. Modulation of dihydropyridine-sensitive calcium channels in heart cells by fish oil fatty acids. Proc Natl Acad Sci U S A 1992;89:1760–4.
30. Pepe S, Bogdanov K, Hallaq H, Spurgeon HA, Leaf A, Lakatta E. Omega 3 polyunsaturated fatty acid modulates dihydropyridine effects on L-type Ca^{2+} channels, cytosolic Ca^{2+}, and contraction in adult rat cardiac myocytes. Proc Natl Acad Sci U S A 1994;91:8832–6.
31. Kang J, Leaf A. Antiarrhythmic effects of polyunsaturated fatty acids: recent studies. Circulation 1996;94:1774–80.

EXHIBIT 9

n−3 Fatty acids and the prevention of coronary atherosclerosis[1,2]

Clemens von Schacky

ABSTRACT Epidemiologic studies have shown an inverse correlation between consumption of fish or other sources of dietary n−3 fatty acids and cardiovascular events. Numerous mechanisms of action for the favorable effect of dietary n−3 fatty acids on factors implicated in the pathogenesis of atherosclerosis have been described. Studies in dogs, swine, and nonhuman primates have consistently shown beneficial effects in various models of vasoocclusive diseases. Studies published currently do not indicate that dietary n−3 fatty acids prevent restenosis after percutaneous coronary angioplasty or induce regression of coronary atherosclerosis. However, in a recent study, occlusion of aortocoronary venous bypass grafts was reduced after 1 y by daily ingestion of 4 g fish-oil concentrate. In the Diet and Reinfarction Trial, 2-y overall mortality was reduced by 29% in survivors of a first myocardial infarction after consumption of n−3 fatty acid–rich fatty fish at least twice a week had been advised (*Lancet* 1989;2:757–61). When n−3 fatty acids were integrated into a diet resembling a traditional Mediterranean diet, 5-y cardiovascular mortality after a first myocardial infarction was reduced by 70% (*Lancet* 1994; 343:1454–9). Preliminary studies indicate that cardiac transplant patients could be an interesting focus of investigation. Currently, food sources rich in n−3 fatty acids are thought to be beneficial in secondary prophylaxis after a myocardial infarction. Large-scale clinical studies with endpoints such as morbidity and mortality are needed to more precisely define the role of n−3 fatty acids in primary and secondary prophylaxis of coronary atherosclerosis. *Am J Clin Nutr* 2000; 71(suppl):224S–7S.

KEY WORDS n−3 Fatty acids, coronary atherosclerosis, primary prevention, secondary prevention, vascular disease, coronary artery disease, myocardial infarction, fish oil

EPIDEMIOLOGIC STUDIES

Epidemiologic studies relating intake of n−3 fatty acids with reduced all-cause mortality continue to be published. Recent evidence of such a relation is discussed elsewhere in this supplement (1). However, as early as 1953 there was curiosity about the diet and disease patterns of the Eskimos (reported in 2). More thorough investigations found surprisingly low cardiovascular mortality in Eskimos consuming a traditional diet rich in eicosapentaenoic (20:5n−3) and docosahexaenoic (22:5n−3) acids (2). The seminal findings in Eskimos were confirmed and extended in Western populations. In most studies, a dose-related, inverse correlation between the intake of fish or n−3 fatty acids and total mortality or cardiovascular mortality was shown (2–4).

MECHANISMS OF ACTION

A comprehensive review of the mechanisms of action described for n−3 fatty acids is beyond the scope of this discussion. Furthermore, data generated in vitro and in animal systems have at times conflicted with those in humans. However, it is worthwhile to note the mechanisms of action related to coronary atherosclerosis suggested by the following results of n−3 fatty acid supplementation studies: *1*) platelet aggregation, as assessed ex vivo in response to various stimuli, is less vigorous after n−3 supplementation (2); *2*) dietary n−3 fatty acids lower triacylglycerol concentrations (5); *3*) large doses of n−3 fatty acids lower cholesterol concentrations (5); *4*) LDL cholesterol appears to increase somewhat dose dependently with n−3 supplementation, the increase apparently being in the size, considered favorable, not the number of molecules (5–7); *5*) increases in HDL-cholesterol concentrations in response to n−3 supplementation were observed in most, but not all, studies (5); *6*) moderate reductions in blood pressure in response to moderate and large doses of n−3 fatty acids were observed consistently (2, 8–10) (a beneficial shift in the eicosanoid system toward vasodilation and antiaggregation is currently thought to contribute to the observed reductions in blood pressure and platelet aggregability; 2); *7*) improvements in rheologic indexes after n−3 supplementation have been observed (2); *8*) more recently, reductions in interleukin 1β and tumor necrosis factor concentrations have been observed ex vivo (11, 12), bringing human cytokine synthesis into focus (11); *9*) A more prominent role in the pathogenesis of atherosclerosis is ascribed to the platelet-derived growth factor (PDGF; 13), *10*) dietary n−3 fatty acids down-regulate gene expression of both PDGF-A and PDGF-B in quiescent mononuclear cells of volunteers (14), indicating that a deliberate change in the diet can change human gene expression; *11*) These findings were confirmed and extended to human mononuclear cells stimulated by adherence (15).

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A consequence—at times catastrophic—of coronary atherosclerosis is cardiac arrhythmias. Although each of the above described mechanisms for n−3 fatty acids by itself may not be sufficiently active to retard atherosclerosis or prevent its catastrophic sequelae, their joint activity may very well be.

STUDIES IN ANIMAL MODELS

In canines, swine, and nonhuman primates, studies with dietary n−3 fatty acids bore consistently positive results in all models of atherosclerosis and vasoocclusion investigated (16). However, results of studies in rats and rabbits have been inconsistent (16). Apparently, there are differences between species with respect to the effect of dietary n−3 fatty acids on models of atherosclerosis. Thus, it does not seem prudent to extrapolate findings in animal models to humans. Therefore, animal models will not be discussed further here.

HUMAN INTERVENTION STUDIES

Prevention of restenosis after percutaneous transluminal coronary angiography

The primary focus of many studies was the prophylaxis of restenosis after a percutaneous transluminal coronary angiography (PTCA; 16–18). On the whole, the first 7 studies conducted showed a favorable effect of dietary n−3 fatty acids (reviewed in 17). However, in subsequent and, more importantly, larger studies, no effect was detected (18, 19). Thus, fish oils are not currently considered to be effective in the prevention of restenosis after PTCA. Nevertheless, the question remains: Why are some studies positive and others negative in their results? Of note, 2 large studies with negative results used corn oil as a placebo, whereas others used olive oil or no placebo. The high rate of restenosis reported for corn oil, however, argues against an effect of corn oil obscuring the effect of n−3 fatty acids (18, 19). Pretreatment was thought to be important for all mechanisms of action to become effective. Although Leaf et al (18) included a minimum of 12 d of pretreatment, the results were negative. However, this question has been raised again by preliminary data of De Caterina et al (20) reported elsewhere in this supplement, pointing to an effect after a 1-mo pretreatment period. Dose-response, or better, incorporation-response analyses of n−3 fatty acids have not yet been conducted to my knowledge, and could yield information for the planning of future studies. The definition of restenosis differs from study to study, reflecting unresolved methodologic problems with quantitative coronary angiography (21, 22). However, a study relying on intravascular ultrasound to assess outcome has not been performed to my knowledge.

A long-held belief was that the prevailing mechanism of restenosis is a growth factor–dependent, rapid proliferation of subendothelial cells (18, 19). Coronary stents (intravascular prostheses made of a metal mesh) have been shown to reduce rates of restenosis from ≈40–50% to ≈20–25% (23). The lower rates of restenosis after placement of intracoronary stents argue against proliferation of subendothelial cells being the only mechanisms of restenosis after PTCA, because stents are not insurmountable obstacles to this proliferation. The success of stents, therefore, may be a result of the prevention of other mechanisms such as retractive processes and scar formation. Because of their success and relative ease of placement, stents will probably obtain a secure place in invasive cardiology. Thus, future strategies toward prevention of restenosis will probably aim toward the reduction of intimal growth in stents. Circumstantial evidence indicates that this process might be regulated by growth factors (13). n−3 Fatty acids had a mitigating effect on some of these growth factors (14, 15). This evidence taken together, the more select group of stented patients might be a better group in which to study prevention of restenosis after PTCA with n−3 fatty acids.

Progression of coronary atherosclerosis

The effect of dietary n−3 fatty acids on the progression or regression of coronary atherosclerosis has been investigated only twice. Sacks et al (24) reported a 2-y study in patients with angiographically documented coronary heart disease and normal plasma lipid concentrations (24). Forty-one patients (of whom 31 completed the trial) were randomly assigned to receive 6 g n−3 fatty acids from fish oil/d and 39 patients (of whom 28 completed the trial) received olive oil in identical capsules. The primary endpoint was the change in minimal luminal diameter, as assessed by quantification of coronary angiograms by a single operator blinded to treatment. In the fish-oil and olive oil groups, 179 and 126 coronary lesions were analyzed, respectively. The change in minimal luminal diameter was 0.104 mm in the fish-oil group and 0.138 mm in the olive oil group (NS). Forty percent of the coronary lesions investigated had a bypass, rendering the study population somewhat heterogenous.

Preliminary results of our own trial, the Study on Prevention of Coronary Atherosclerosis by Intervention with Marine Omega-3 Fatty Acids (SCIMO) have been published in abstract form (25). We tested the hypothesis that consumption of a fish-oil concentrate for 2 y would reduce a score to assess progression and regression of coronary atherosclerosis, as assessed by coronary angiography, by 50%. Identical opaque capsules contained either n−3 fatty acids (55% eicosapentaenoic and docosahexaenoic acids) or a placebo reflecting the average fatty acid composition of the European diet. The capsule contents weighed 1 g each, and the patients were instructed to ingest 6 capsules/d for the first 3 mo and 3 capsules/d for the subsequent 21 mo. The study was approved by the ethics committee of the medical faculty of the University of Munich and was conducted according to *Good Clinical Practice*, the pertinent guidelines of the European Union. From September 1992 to June 1994, 223 patients with mild coronary atherosclerosis gave informed consent and were recruited. Patient compliance was monitored by red blood cell phospholipid fatty acid analyses. Forty-nine patients did not complete the study, and follow-up coronary angiography was not performed on 12 patients who did complete the study. No patients were lost to follow-up. The code was broken after deposition of all relevant data at the trial monitor. One hundred eleven and 112 patients received n−3 fatty acids and placebo capsules, respectively. Randomization was successful with respect to age, sex, risk factors, lipid-lowering therapy, and other indexes. Analysis of all other results is being performed as of this writing in 1997.

Only one trial has addressed the question of whether n−3 fatty acids had an effect on coronary bypass patency 1 y after surgery (26). On the first postoperative day, 317 patients were randomly assigned to supplement their diet with 4 g 83% n−3 fatty acid concentrate/d; 293 patients received no supplement. Follow-up angiograms were performed on 302 patients in the n−3 fatty acid group and on 279 in the control group. The patency of internal

mammaria grafts was not affected. However, of 635 and 595 distal anastomoses of venous bypass grafts, 174 and 196 were occluded in the n−3 fatty acid and control groups, respectively. This gave the patients receiving n−3 fatty acids an odds ratio of 0.77 (95% CI: 0.60, 0.99; $P = 0.034$) of distal anastomosis occlusion compared with the control group (26). Forty-three percent of the patients who received n−3 fatty acids had at least one vein graft occluded compared with 51% of the control patients ($P = 0.05$). n−3 Serum phospholipid concentration and response analysis showed a positive dose-response effect of n−3 fatty acids.

Few patients died in the studies mentioned previously, obviating a life table analysis for all studies combined. Unfortunately, definitions of other clinical endpoints, as well as the methods of reporting them, varied. Thus, a meaningful meta-analysis of the clinical course of the patients recruited for the studies cited is not possible from the published reports (17–19, 24–26).

Survival after myocardial infarction

The clinical course of survivors of a first myocardial infarction was investigated by the Diet and Reinfarction Trial (DART; 27). In a factorial randomized design, 2033 men either received or did not receive advice to alter their diet on average 41 d after the index event. Three recommendations were given. The first group was advised to reduce dietary fat and its composition according to the American Heart Association Step I diet. The second group was advised to eat fatty fish 2–3 times/wk or, for those unable to tolerate fish, to supplement their diet with fish-oil capsules, providing 0.5 g n−3 fatty acids/d. In this group, plasma eicosapentaenoic acid concentrations increased to 0.59% ($P < 0.01$ compared with the control group). The third group was advised to eat 18 g fiber/d. After 2 y, the patients given the fish advice had a 29% reduction in total mortality compared with the patients given no such advice (27). Interestingly, this reduction was not associated with a sustained reduction in serum cholesterol in the patients advised to consume fish or fish oil. Thus, the results of DART cannot be explained by extrapolation of similar results in trials in which cholesterol-lowering approaches were used (28). The authors speculated that the main reason for the 29% reduction in mortality was a reduced incidence of ventricular fibrillation during reinfarctions or some other acute myocardial ischemia. In the group advised to consume fish or fish oil, the incidence of nonfatal myocardial infarctions was higher, but not significantly so, than in the group given no advise. Thus, these data would also be consistent with smaller, and thus less lethal, infarctions occurring in the group advised to consume fish or fish oil. The results of DART have stimulated researchers of n−3 fatty acids. The fact that little money has been available for clinical studies with n−3 fatty acids in the cardiovascular area has, however, precluded confirmation of the results of DART.

Nevertheless, the results of the Lyon study (29) can, in a very broad sense, be regarded as supporting DART's results. In a randomized study in patients similar to those studied in DART, subjects were given multifaceted dietary advice plus a margarine specifically designed to reflect a traditional Mediterranean diet. This diet incorporated an n−3 fatty acid [in this case α-linolenic acid (18:3n−3)] and consisted predominantly of vegetables and fruit with some white meat, but no red meat, included. Plasma fatty acid concentrations were analyzed in 141 of the 302 experimental patients and in 139 of 303 control patients. In the experimental group, α-linolenic acid increased to 0.62%, eicosapentaenoic acid increased to 1.03% ($P < 0.001$), and cardiovascular mortality was 70% lower at 5 y compared with the control group (29). Total cholesterol and LDL-cholesterol concentrations were identical in the control and experimental groups, pointing to effects independent of these 2 indexes.

Cardiac transplantation

In cardiac transplant patients, endothelium-dependent coronary vasodilation, as assessed after acetylcholine infusion, was normal after 3 wk of consuming 5 g dietary n−3 fatty acids/d, whereas it was pathologic in otherwise matched control subjects (30). This could contribute to the inhibition of transplant vasculopathy and longer cardiac graft survival seen in animal models of cardiac transplantation (31, 32). Whether n−3 fatty acids have an influence on cardiac transplant survival in humans is currently unknown.

Peripheral artery disease

In animal models of peripheral artery disease, beneficial effects of n−3 fatty acids have been shown in dogs, swine, and nonhuman primates (16). Studies in humans with clinical endpoints such as pain-free distance walking have been scarce and have shown no benefit of n−3 fatty acid consumption (16). Results derived from our ultrasound studies are pending. Arterial compliance seems to be improved after 6 wk of n−3 fatty acid supplementation in diabetic patients (33). Forearm vasoconstrictory response to pressors is reduced after ingestion of n−3 fatty acids, an effect thought to be mediated by the eicosanoid system (34). The effects of n−3 fatty acids on peripheral artery disease are much less well studied than are those on coronary artery disease. As yet, no clear-cut clinical benefit on peripheral arteries can be discerned.

CONCLUSION

In coronary artery disease, consumption of dietary n−3 fatty acids has been found to reduce mortality after a first myocardial infarction. Although confirming studies are needed, dietary n−3 fatty acids appear to be of value in secondary prevention of coronary artery disease. At present, we have no evidence that dietary n−3 fatty acids induce regression of coronary atherosclerosis. Ingestion of n−3 fatty acids appears to reduce occlusion of coronary venous bypass grafts. An interesting focus of research is on heart transplant patients. Large-scale clinical studies with endpoints such as morbidity and mortality are needed to more precisely define the role of n−3 fatty acids in primary and secondary prevention of coronary atherosclerosis.

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REFERENCES

1. Siscovick DS, Raghunathan TE, King I, et al. Dietary intake of long-chain n−3 polyunsaturated fatty acids and the risk of primary cardiac arrest. Am J Clin Nutr 2000;71(suppl):208S–12S.
2. von Schacky C. Prophylaxis of atherosclerosis with marine omega-3 fatty acids—a comprehensive strategy. Ann Intern Med 1987; 107:890–9.
3. Dolecek TA. Epidemiological evidence of relationships between dietary polyunsaturated fatty acids and mortality in the multiple risk factor intervention trial. Proc Soc Exp Biol Med 1992;200: 177–82.
4. Ascherio A, Rimm EB, Stampfer MJ, Giovannucci EL, Willett WC. Dietary intake of marine n−3 fatty acids, fish intake, and the risk of coronary disease among men. N Engl J Med 1995;332:977–82.

5. Harris WS. Fish oils and plasma lipid and lipoprotein metabolism in humans: a critical review. J Lipid Res 1989;30:785–807.
6. Axelrod L, Camuso J, Williams E, Kleinman K, Briones E, Schoenfeld D. Effects of a small quantity of omega-3 fatty acids on cardiovascular risk factors in NIDDM—a randomized, prospective, double-blind, controlled study. Diabetes Care 1994;17:37–44.
7. Suzukawa M, Abbey M, Howe PRC, Nestel PJ. Effects of fish oil fatty acids on low density lipoprotein size, oxidizability, and uptake by macrophages. J Lipid Res 1995;36:473–84.
8. Knapp HR, FitzGerald GA. The antihypertensive effects of fish oil. A controlled study of polyunsaturated fatty acid supplement in essential hypertension. N Engl J Med 1989;320:1037–43.
9. Bønaa KH, Bjerve KS, Straume B, Gram IT, Thelle D. Effect of eicosapentaenoic and docosahexaenoic acids on blood pressure in hypertension. A population-based intervention trial from the Tromsø study. N Engl J Med 1990;322:795–801.
10. Toft I, Bonaa KH, Ingebretsen OC, Nordoy A, Jenssen T. Effects of n−3 polyunsaturated fatty acids on glucose homeostasis and blood pressure in essential hypertension—a randomized, controlled trial. Ann Intern Med 1995;123:911–8.
11. Endres S, von Schacky C. n−3 Polyunsaturated fatty acids and human cytokine synthesis. Curr Opin Lipidol 1996;7:48–52.
12. Endres S, Ghorbani R, Kelley VE, et al. The effect of dietary supplementation with n−3 polyunsaturated fatty acids on the synthesis of interleukin-1 and tumor necrosis factor by mononuclear cells. N Engl J Med 1989;320:262–71.
13. Ross R. Pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 1993;362:801–9.
14. Kaminski WE, Jendraschak E, Kiefl R, von Schacky C. Dietary omega-3 fatty acids lower levels of platelet-derived growth factor messenger RNA in human mononuclear cells. Blood 1993;81:1871–9.
15. Baumann KH, Hessel F, Larass I, et al. Dietary omega-3, omega-6, and omega-9 unsaturated fatty acids and growth factor and cytokine gene expression in unstimulated and stimulated monocytes. A randomized volunteer study. Arterioscler Thromb Vasc Biol 1999; 19:59–66.
16. von Schacky C. Cardiovascular effects of n−3 fatty acids. In: Frölich JC, von Schacky C, eds. Fish, fish oil and human health. Munich, Germany: Zuckschwerdt Verlag, 1992:167–78.
17. Gapinski JP, VanRuiswyk JV, Heudebert GR, Schectman GS. Preventing restenosis with fish oils following coronary angioplasty. A meta-analysis. Arch Intern Med 1993;153:1595–601.
18. Leaf A, Jorgensen MB, Jacobs AK, et al. Do fish oils prevent restenosis after coronary angioplasty? Circulation 1994;90:2248–57.
19. Cairns JA, Gill J, Morton B, et al. Fish oils and low-molecular-weight heparin for the reduction of restenosis after percutaneous transluminal coronary angioplasty. The EMPAR study. Circulation 1996;94: 1553–60.
20. De Caterina R, Liao JK, Libby P. Fatty acid modulation of endothelial activation. Am J Clin Nutr 2000;71(suppl):213S–23S.
21. Hong MK, Mintz GS, Popma JJ, et al. Limitations of angiography for analyzing coronary atherosclerosis progression or regression. Ann Intern Med 1994;121:348–54.
22. Keane D, Haase J, Slager CJ, et al. Comparative validation of quantitative coronary angiography systems. Results and implications from a multicenter study using a standardized approach. Circulation 1995;91: 2174–83.
23. Eeckhout E, Kappenberger L, Goy JL. Stents for intracoronary placement: current status and future directions. J Am Coll Cardiol 1996;27:757–65.
24. Sacks FM, Stone PH, Gibson CM, Silverman DI, Rosner B, Pasternak RC. Controlled trial of fish oil for regression of human coronary atherosclerosis. J Am Coll Cardiol 1995;25:1492–8.
25. von Schacky C, Angerer P, Kothny W, Theisen K, Mudra H. Study on Prevention of Coronary Atherosclerosis with Marine Omega-3 fatty acids (SCIMO)—first results of a randomized double blind study. Can J Cardiol 1997;13(suppl):42B (abstr).
26. Eritsland J, Arnesen H, Gronseth K, Fjeld NB, Abdelnoor M. Effect of dietary supplementation with n−3 fatty acids on coronary artery bypass graft patency. Am J Cardiol 1996;77:31–36.
27. Burr ML, Fehily AM, Gilbert JF, et al. Effects of changes in fat, fish, and fibre intakes on death and myocardial reinfarction: Diet and Reinfarction Trial. Lancet 1989;2:757–61.
28. The Scandinavian Simvastatin Survival Study Group. Randomized trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). Lancet 1994;344:1383–9.
29. de Lorgeril M, Renaud S, Mamelle N, et al. Mediterranean alpha-linolenic acid–rich diet in secondary prevention of coronary heart disease. Lancet 1994;343:1454–9.
30. Fleischhauer FJ, Yan WD, Fischell TA. Fish oil improves endothelium-dependent coronary vasodilation in heart transplant recipients. J Am Coll Cardiol 1993;21:982–9.
31. Sarris GE, Mitchell RS, Billingham ME, Glasson JR, Cahill PD, Miller DC. Inhibition of accelerated cardiac allograft arteriosclerosis by fish oil. J Thorac Cardiovasc Surg 1989;97:841–55.
32. Grimminger F, Grimm H, Fuhrer D, et al. Omega-3 lipid infusion in a heart allotransplant model: shift in fatty acid and lipid mediator profiles and prolongation of transplant survival. Circulation 1996; 93:365–71.
33. McVeigh GE, Brennan GM, Cohn JN, Finkelstein SM, Hayes RJ, Johnston GD. Fish oil improves arterial compliance in non-insulin-dependent diabetes mellitus. Arterioscler Thromb 1994;14: 1425–9.
34. Chin JPF, Gust AP, Dart AM. Indomethacin inhibits the effects of dietary supplementation with marine oils on vasoconstriction of human forearm resistance vessels in vivo. J Hypertens 1993; 11:1229–34.

EXHIBIT 10

Before the
FOOD AND DRUG ADMINISTRATION
Rockville, MD

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0368

In re: Guidance for Industry:	)	
Significant Scientific Agreement	)	
In the Review of Health Claims	)	Docket No. 99D-5424
For Conventional Foods and	)	
Dietary Supplements; Availability	)	

COMMENTS OF
JULIAN M. WHITAKER, M.D.;
PURE ENCAPSULATIONS, INC.;
XCEL MEDICAL PHARMACY, LTD.;
MYCOLOGY RESEARCH LABORATORIES, LTD.;
DURK PEARSON and SANDY SHAW; and
AMERICAN PREVENTIVE MEDICAL ASSOCIATION

Julian M. Whitaker, M.D.; Pure Encapsulations, Inc.; XCEL Medical Pharmacy, Ltd.; Mycology Research Laboratories, Ltd.; Durk Pearson and Sandy Shaw; and the American Preventive Medical Association (collectively, "Joint Commenters"), hereby submit their comments in response to the agency's solicitation for comments in the above-referenced docket. See 64 Fed. Reg. 71794 (1999).

BACKGROUND OF JOINT COMMENTERS

Julian M. Whitaker, M.D. Julian M. Whitaker, M.D. ("Dr. Whitaker) is a physician licensed to practice medicine in the states of California and Washington. He graduated from Dartmouth College in 1966 with a B.S. degree and from Emory University in 1970 with an M.D. degree. He received additional training in surgery as a resident at the University of California Medical School. From 1975 to 1976 he worked as a physician at the Pritikin Institute in California. Since that time he has been the clinical director of the Whitaker Wellness Institute in Newport Beach, California. He is the author of five books: ***Reversing Heart Disease*** (1985), ***Reversing Diabetes*** (1987),

Reversing Health Risk (1989), ***Natural Healing*** (1994), and ***What Your Doctor Won't Tell You About Bypass*** (1995). Since August of 1991 he has been the editor of ***Health & Healing***, currently the nation's largest single editor health newsletter. In 1996, ***Health & Healing*** had over 500,000 subscribers. He receives royalties from the distribution and sale of several dietary supplements. Dr. Whitaker has filed with FDA several health claim petitions and would like to use the health claims on the labels and in the labeling of dietary supplements. He therefore has a keen interest in how FDA interprets its health claim standard and is adversely affected by FDA's insistence on a standard more rigorous than that intended by Congress.

Durk Pearson and Sandy Shaw. Durk Pearson and Sandy Shaw ("Pearson and Shaw") are scientists residing in Nevada. They design dietary supplement formulations and license them to manufacturing and retailing companies. They are authors of four books on aging and age-related diseases, including the #1, million plus copy best seller ***Life Extension: A Practical Scientific Approach*** (1982). They have also published three other health books, two of which were best sellers: ***The Life Extension Companion*** (1984); ***The Life Extension Weight Loss Program*** (1986); and ***Freedom of Informed Choice—FDA Versus Nutrient Supplements*** (1993). Durk Pearson and Sandy Shaw were plaintiffs in the *Pearson v. Shalala* case that is the subject of these comments. Pearson and Shaw license dietary supplements. They have filed with FDA several health claim petitions and would like to use the health claims on the labels and in the labeling of dietary supplements. They therefore have a keen interest in how FDA interprets its health claim standard and are adversely affected by FDA's insistence on a standard more rigorous than that intended by Congress.

American Preventive Medical Association. The American Preventive Medical Association ("APMA") is a non-profit organization in Virginia. APMA was founded in October of 1992 and is dedicated to ensuring consumer access to preventive therapies and the rights of health care providers to offer those therapies. APMA was a plaintiff in the *Pearson v. Shalala* case that sought FDA approval of four health claims. Several APMA practitioner members sell dietary supplements and would like to use the health claims on the labels and in the labeling of those supplements. APMA practitioner members are desirous of filing additional health claim petitions with FDA. In addition, APMA and its practitioner members and their hundreds of thousands of patients would benefit from an effective and meaningful health claim approval process as described herein because it would enable them to communicate and receive nonmisleading health information on labels and in labeling of dietary supplements. APMA and its members therefore have a keen interest in how FDA interprets its health claim standard and are adversely affected by FDA's insistence on a standard more rigorous than that intended by Congress.

Mycology Research Labs Ltd. Mycology Research Labs Ltd. ("Mycology") is a corporation organized in Great Britain and engaged in the business of manufacturing, distributing, and selling multiple pharmaceutical grade dietary supplements for human consumption around the world, including in the United States. Mycology is desirous of filing with FDA several health claim petitions and would like to use the health claims on the labels and in the labeling of dietary supplements that it manufactures, distributes, and sells in the United States. It therefore has a keen interest in how FDA interprets its health claim standard and is adversely affected by FDA's insistence on a standard more rigorous than that intended by Congress.

Pure Encapsulations, Inc. Pure Encapsulations, Inc. ("Pure") is a Massachusetts corporation engaged in the business of manufacturing, distributing, and selling pharmaceutical grade dietary supplements for human and companion animal consumption. Pure has filed with FDA several health claim petitions and would like to use the health claims on the labels and in the labeling of dietary supplements. It therefore has a keen interest in how FDA interprets its health claim standard and is adversely affected by FDA's insistence on a standard more rigorous than that intended by Congress.

XCEL Medical Pharmacy, LTD d/b/a XCEL Health Care. XCEL Medical Pharmacy, LTD d/b/a XCEL Health Care ("XCEL") is a California corporation engaged in the business of manufacturing, distributing, and selling pharmaceutical grade dietary supplements for human consumption. XCEL is desirous of filing with FDA health claim petitions and would like to use health claims on the labels and in the labeling of dietary supplements that it manufactures, distributes, and sells. It therefore has a keen interest in how FDA interprets its health claim standard and is adversely affected by FDA's insistence on a standard more rigorous than that intended by Congress.

BACKGROUND OF AGENCY NOTICE

In 21 U.S.C. § 343(r)(5(D), Congress assigned the Food and Drug Administration the task of establishing a "procedure and standard respecting the validity of [the health] claim." The FDA, however, did not provide regulatees with a defined standard for review of health claims. On January 15, 1999, the United States District Court for the District of Columbia held the FDA's failure to define a standard for dietary supplement health claims a violation of the Administrative Procedure Act (APA). *Pearson v.*

Shalala, 164 F.3d 650, 659-661 (D.C. Cir.1999), *reh'g denied en banc,* 172 F.3d 72 (D.C. Cir. 1999).

In particular, the Court held FDA's failure to give definitional content to the phrase "significant scientific agreement" (its lode stone in reviewing dietary supplement health claims) a violation of the APA's prohibition on arbitrary and capricious agency action. *Pearson,* 164 F.3d at 660-661. The Court reasoned that "[i]t simply will not do for a government agency to declare—without explanation—that a proposed course of private action is not approved." It further reasoned that "[t]o refuse to define the criteria [the agency] is applying is equivalent to simply saying no without explanation." *Id.*

The Court held that FDA was required either case by case or sub-regulation by sub-regulation to define the standard, to "explain what [FDA] means by significant scientific agreement or, at minimum, what it does not mean." *Pearson,* 164 F.3d at 661. The Court required FDA to define the standard in a manner that would make it "possible for the regulated class to perceive the principles which are guiding agency action." *Id.*.

The Court explained that it could be possible for FDA to define a standard with sufficient particularity that would satisfy the Administrative Procedure Act but yet not define it with that degree of particularity required to satisfy the First or Fifth Amendments to the United States Constitution. *Pearson,* 164 F.3d at 660 n.12.

On December 22, 1999, the FDA responded to the APA holding in the *Pearson* Court's remand not by promulgating a new rule but by issuing a notice of a guidance. 64 Fed. Reg. 71794 (Dec. 22, 1999). In its Guidance, FDA explains that it reviews "all relevant studies" concerning the nutrient/disease relationship and does so under a hierarchy that deems interventional studies involving randomized, controlled clinical

trials as the "gold standard." Guidance at 4-5. Next down from the randomized, controlled clinical trials are observational studies, with greater preference accorded prospective than retrospective studies. Observational studies are, themselves, given a hierarchy: (1) cohort (longitudinal) studies; (2) case-control studies; (3) cross-sectional studies; (4) uncontrolled case series or cohort studies; (5) time-series studies; (6) ecological or cross-population studies; (7) descriptive epidemiology; and (8) case reports. Below observational studies are the following in their order of relative weight and significance: (1) research synthesis studies and (2) animal and in vitro studies. Guidance at 5.

The agency next discusses its method for ascertaining whether the studies include reliable measures of the substance and the disease or health-related condition. Guidance at 7. FDA states that it must identify "biomarkers (immediate or surrogate endpoint markers) for the presence or risk of disease." Guidance at 7. FDA states that it must be able to identify and measure the substance in a food and determine the impact of that measured substance on the disease or health-related condition exclusive of other dietary components or the food itself. Guidance at 8-9.

In evaluating scientific studies, FDA will assess the susceptibility of the study to bias and confounders; quality assessment criteria (including adequacy and clarity of design; population studied; analytical methodology and quality control procedures); and the statistical methods used. Guidance at 10-13.

In evaluating the totality of the scientific evidence, FDA requires proof that "a change in the dietary intake of the substance ***will*** result in a change in a disease endpoint." Guidance at 13 (emphasis added). Moreover, it requires proof of causation,

demanding strong evidence of a causal relationship. Guidance at 14-15. The agency depends primarily on use of interventional studies (randomized, controlled clinical trials) as a condition precedent to proof of causation, writing:

> Causality can be best established by interventional data, particularly from randomized, controlled clinical trials, that show that altering the intake of an appropriately identified and measured substance results in a change in a valid measure of a disease or health-related condition. In the absence of such data, a causal relationship may be inferred based on observational and mechanistic data through strength of association, consistency of association, independence of association, dose-response relationship, temporal relationship, effect of dechallenge, specificity, and explanation of a pathogenic mechanism or a protective effect against such a mechanism (biological plausibility). Although these features strengthen the claim that a substance contributes to a certain health outcome, they do not prove that eating more or less of the substance will produce a clinically meaningful outcome. In many cases (for example, if the intake of the substance has not been or cannot be assessed adequately in available observational studies because it has not been commonly consumed or its intake cannot be assessed independently of other substances), controlled clinical trials are necessary to establish the validity of a substance/disease relationship.

Guidance at 15.

In determining the weight of the scientific evidence, FDA requires that two questions be answered in the affirmative: (1) whether the evidence in support of the substance/disease relationship outweighs that against it and (2) whether the evidence corroborates "that a change in the dietary intake of the substance ***will*** result in a change in the disease endpoint." Guidance at 16 (emphasis added).

In the all-important matter of defining "significant scientific agreement," FDA states that "[i]n the process of scientific discovery, significant scientific agreement occurs well after the state of emerging science, where data and information permit an inference, but before the point of unanimous agreement within the relevant scientific community that the inference is valid." Guidance at 16. The agency states that "significant scientific agreement is not consensus in the sense of unanimity, it represents considerably more

than an initial body of emerging evidence." Guidance at 16-17. In assessing whether significant scientific agreement exists, FDA states that it will "take[] into account the viewpoints of qualified experts outside the agency. . ." Guidance at 18. It states that it will "take into account:

> • *review publications that critically summarize data and information in the secondary scientific literature;*
> • *documentation of the opinion of an "expert panel" that is specifically convened for this purpose by a credible, independent body;*
> • *the opinion or recommendation of a federal government scientific body such as the National Institutes of Health (NIH) or the Centers for Disease Control and Prevention (CDC); or the National Academcy of Sciences (NAS); or an independent, expert body such as the Committee on Nutrition of the American Academy of Pediatrics (AAP), the American Heart Association (AHA), American Cancer Society (ACS), or task forces or other groups assembled by the National Institutes of Health (NIH).*

Guidance at 18.

SUMMARY

The United States Court of Appeals' mandate to FDA is to "explain what [FDA} means by significant scientific agreement or, at minimum, what [FDA] does not mean." *Pearson*, 164 F.3d at 661. The Guidance fails to comply with the mandate. While in the Guidance FDA has listed the rank it accords to varying types of scientific evidence (without specifying the comparative or cumulative weight of the different kinds of evidence) and has indicated that it expects near conclusive proof of causality as a condition precedent to claim approval, it has avoided explaining what it means by significant scientific agreement; it has also avoided explaining what it does not mean.

The Court's mandate asks FDA to provide the regulated class sufficient information "to perceive the principles which are guiding agency action." The Guidance does not provide information necessary for regulatees to perceive FDA's guiding

principles. It does not explain the meaning of significant scientific agreement. While, from the Guidance, the regulated class can understand that FDA views interventional studies involving well designed randomized, controlled clinical trials as its "gold standard," it is entirely impossible from the Guidance to perceive whether FDA will ever accept studies other than interventional or other than those involving randomized, controlled clinical trials as sufficient for claim authorization. It appears unlikely that FDA ever will because it requires proof of direct causality. Given FDA's insistence on proof of direct causality (that a substance ***will*** result in a change in a disease endpoint) as a condition precedent to claim approval, it appears that only claims backed by well designed randomized, controlled clinical trials coupled with proof of direct causality will cause FDA to permit claim authorization. A large body of evidence strongly supporting, but not conclusively proving, a substance-disease relationship appears unlikely to satisfy the FDA.

Thus, the only principle that regulatees can perceive with clarity from FDA's Guidance is that FDA will accept the same kind of near conclusive proof expected as a condition precedent for drug approval as a condition precedent for dietary supplement claim approval. That principle violates Congressional intent, however. Congress plainly expects this agency to authorize health claims for dietary supplements without requiring that those claims be backed by the same kind of near conclusive proof required for the grant of applications for new drugs. Accordingly, to the extent that FDA's Guidance reveals a principle to the regulated class, that principle is one calling for a level of evidence that Congress has unequivocally rejected in the context of health claims for dietary supplements.

In addition, FDA's Guidance includes an unscientific bias and favoritism for certain non-governmental organizations, namely the Committee on Nutrition of the American Academy of Pediatrics, the American Heart Association, and the American Cancer Society. The agency places special emphasis upon the opinions and recommendations of these private organizations equating the value of those with the opinions and recommendations of federal government scientific bodies. It omits from specific reference the opinions and recommendations of other private bodies, such as universities, professional and scientific associations, and other scientific authorities. The action reveals an unscientific bias in favor of the private organizations listed and an arbitrary and capricious grant of privilege to the named private organizations to the exclusion of all others.

Finally, FDA's Guidance omits reference to the constitutional mandate in *Pearson*. The Guidance misleads the public and the regulated class to the extent that it suggests that a dietary supplement health claim not approved by FDA under its "significant scientific agreement" standard is prohibited on labels and in labeling. Under *Pearson*'s constitutional mandate, even if claims fail the "significant scientific agreement" test, FDA must nevertheless authorize all that are, at worst, potentially misleading with corrective disclaimers. *Pearson*, 164 F.3d at 659-660. Because the constitutional mandate interprets the First Amendment to the United States Constitution and the First Amendment is the higher law against which contrary law cannot stand, FDA must make clear to the regulated class within the Guidance that a claim it deems not backed by "significant scientific agreement" will nevertheless be authorized when a disclaimer can render it nonmisleading.

For these reasons, explained in detail below, FDA should promptly revise its Guidance. It should comply with the mandate of the United States Court of Appeals for the D.C. Circuit by explaining what it means by significant scientific agreement or, at minimum, what it does not mean. In that regard, FDA cannot rest upon the highly inexact and largely vacuous and variable statement that significant scientific agreement occurs after emerging science but before unanimous agreement. The universe described is immense, so immense as to exceed any reasonable definitional boundary. Indeed, nearly all scientific evidence falls between the polar extremes of emerging science and consensus. Accordingly, FDA should define with as much specificity as possible where on the continuum of scientific evidence between emerging science and consensus "significant scientific agreement" lies. Does it occur when a significant minority or segment of scientists who study the relationship agree that the claimed relationship is supported by the scientific evidence? Does it occur when at least half of the scientists who study the relationship agree that the claimed relationship is supported by the scientific evidence? Does it occur when at least three quarters of the scientists who study the relationship agree that the claimed relationship is supported by the scientific evidence? When may it be said on the continuum of scientific evidence that significant scientific agreement has been reached? In that regard, consistent with the dictates of Congress, FDA should hold that significant scientific agreement exists when

> a significant segment of scientists having relevant expertise agree, based on relevant scientific evidence, that consumers are *reasonably likely* to obtain the claimed health benefit.

Senate Report 103-410, at 24.

Congress determined that the above-quoted definition it supplied in committee is "consistent with the NLEA's goal of assuring that consumers have access on food and dietary supplement labels to health claims that are scientifically supported, without having to wait until the degree of scientific certainty contemplated by the drug standard has been achieved." *Id.* FDA's insistence on a higher standard, the equivalent of the drug certainty standard used as a condition precedent to grant of applications for new drugs, conflicts with Congress's intentions and cannot stand.

ARGUMENT

A. FDA'S GUIDANCE VIOLATES *PEARSON*'S APA MANDATE BY FAILING TO DEFINE "SIGNIFICANT SCIENTIFIC AGREEMENT"

The *Pearson* Court ordered FDA to "explain what it means by significant scientific agreement or, at minimum, what it does not mean." *Pearson,* 164 F.3d at 661. FDA's Guidance fails to comply. Nowhere in the entire Guidance does FDA provide any reasonable explanation of what it means by significant scientific agreement (or what it does not mean). The only "definition" for the term that the agency offers in the Guidance is one so broad, so vacuous, and so inexact as to be entirely unusable by the regulated class. Indeed, the extraordinary breadth of the definition suggests that any meaning FDA imparts to the term on a case by case basis may be the product of political discretion (or anti-dietary supplement bias) as much, if not more, than rational scientific judgment. In the Guidance, the agency states that, "[i]n the process of scientific discovery, significant scientific agreement occurs well after the state of emerging science, where data and information permit an inference, but before the point of unanimous agreement within the relevant scientific community that the inference is valid." Guidance at 16. That language embraces nearly the entire body of scientific evidence and does not afford the regulated

class sufficient information to discern where along the continuum of science between emerging data and consensus the point of significant scientific agreement exists. With the agency's definition, the regulated class certainly cannot discern the principles which guide FDA action (except that satisfaction of the drug certainty standard will probably suffice). Accordingly, the definition violates *Pearson*'s APA mandate to the agency. To comply with the mandate, FDA must revise its Guidance promptly as explained below.

B. FDA'S GUIDANCE VIOLATES *PEARSON*'S APA MANDATE BY NOT REVEALING THE PRINCIPLES WHICH GUIDE AGENCY ACTION ON CLAIMS SUPPORTED BY EVIDENCE OTHER THAN INTERVENTIONAL STUDIES BEARING PROOF OF DIRECT CAUSALITY

From the Guidance, one may discern that FDA has adopted a hierarchy to evaluate scientific evidence, placing at its top well designed interventional studies (and at the top of such studies randomized, controlled clinical trials). Although FDA's preference for well designed interventional studies is reiterated throughout the document, the FDA does not explain whether studies other than the very lengthy and expensive randomized, controlled interventional ones will suffice and, if other studies would, what comparative and cumulative weight FDA affords evidence other than randomized, controlled interventional studies. For example, from the Guidance it is impossible to determine whether FDA would ever accept as a substitute for randomized, controlled interventional studies, a combination of observational and mechanistic studies, or—if so—what kind of such studies would suffice to substitute for randomized, controlled interventional studies.

From the Guidance, one may discern that FDA demands that the regulated class supply it with proof that "a change in the dietary intake of the substance ***will*** result in a

change in a disease endpoint." FDA thus calls for conclusive proof of causality. FDA expects conclusive proof of causality regardless of the nature of the claim. Thus, a claim that a nutrient "may" reduce the risk of a disease or "may" reduce the symptoms of a disease is treated in the same manner as one that states a direct causal relationship (e.g., nutrient X will reduce the risk of disease Y, or nutrient X will reduce the symptoms of disease Y). Direct proof of causality is equal to that degree of proof required by this agency, pursuant to the "substantial evidence" standard, as a condition precedent to the grant of applications for new drugs. 21 U.S.C. § 355(e) (see generally *Weinberger v. Hynson Westcott & Dunning, Inc.*, 412 U.S. 609 (1973) and *E.R. Squibb & Sons, Inc. v. Bowen*, 870 F.2d 678, 679 (D.C. Cir. 1989).

FDA states that in evaluating the scientific evidence, it will require an affirmative answer to the following two questions: (1) whether the evidence in support of the substance/disease relationship outweighs that against it and (2) whether the evidence corroborates "that a change in the dietary intake of the substance ***will*** result in a change in the disease endpoint." Thus, in light of FDA's clear preference for randomized, controlled clinical trials and its insistence on direct evidence of causality, to the extent that a principle can be discerned from the Guidance, it is that FDA will authorize claims upon receipt of proof that they are corroborated by randomized, controlled clinical trials and upon receipt of proof of direct causality. That kind of near conclusive proof is the same as that required by FDA for approval of new drug applications. Accordingly, to the extent that FDA's Guidance reveals a principle to the regulated class it is one calling for a level of evidence Congress has unequivocally rejected in the context of health claims for dietary supplements. FDA must revise its Guidance. It must replace it with one that

complies with *Pearson*'s APA order and the dictates of Congress on interpreting "significant scientific agreement." The current Guidance fails on both accounts.

C. FDA'S GUIDANCE HARBORS AN UNSCIENTIFIC BIAS AND FAVORITISM FOR CERTAIN PRIVATE ORGANIZATIONS

In addition to its failure to explain what significant scientific agreement means (or, conversely, what it does not mean) in a manner that can enable the regulated class to discern the principles which guide agency action, the Guidance includes specific reference to a select group of private organizations. The reference gives equal weight to the opinions and recommendations of those organizations and the opinions and recommendations of federal government scientific bodies. Moreover, it fails to give equivalent weight to the opinions and recommendations of any other scientific body, e.g., any or all universities, other private scientific associations, and recognized authorities in the field of science. The agency offers no explanation for why the named private organizations (Committee on Nutrition of the American Academy of Pediatrics; the American Heart Association; and the American Cancer Society) should be given preferential treatment and status in the evaluation of health claims. For example, it does not explain (nor could it reasonably) why these private associations in particular are possessed of scientific insights, knowledge, and evidence superior to all others or why these private associations in particular should be viewed as equivalent to federal government scientific bodies. It is not at all unworthy of note that the American Heart Association and the American Cancer Society were amicus curiae in favor of the unsuccessful position articulated by the FDA in the *Pearson* case. Through that relationship, let alone all others between the FDA and those groups, FDA has engaged in legal and political battle against authorization of dietary supplement health claims. Thus,

far from serving as an unbiased source for opinion and recommendation, FDA has chosen precisely those entities that have a track record of partisan support for FDA's positions. For these many reasons, FDA's select listing of preferred private organizations in the Guidance constitutes arbitrary and capricious agency action and should be reversed in print as well as deed. The Joint Commenters do not object to agency acceptance of the opinion and recommendations of private scientific associations as sources of reputable information relevant to the evaluation of supplement-disease relationships, but the Joint Commenters strongly object to the arbitrary and capricious limited selection of three named associations made in the Guidance by FDA.

D. FDA'S GUIDANCE IS MISLEADING BECAUSE IT OMITS REFERENCE TO *PEARSON*'S CONSTITUTIONAL STANDARD AS AN ALTERNATIVE GROUND FOR AUTHORIZATION

The Director of the Center for Food Safety and Applied Nutrition has made it clear that FDA understands *Pearson*'s constitutional mandate to necessitate agency authorization of health claims even when those claims fail to satisfy its "significant scientific agreement" standard. Director Levitt wrote:

> . . . [W]e agree that the court's decision requires FDA to reconsider not only whether each of the four claims meets the significant scientific agreement standrd, but also, even if that standard is not met, whether the addition of a disclaimer to the claim could render it non-misleading. If the answer to either question is yes, we will authorize the claim.

See Exhibit A.

Indeed, the *Pearson* decision's constitutional mandate takes primacy over contrary agency rules and interpretations. It is, after all, the First Amendment which, under the Supremacy Clause, is the supreme law of the land. U.S.CONST. Art. VI. See also *Marbury v. Madison*, 5 U.S. 137, 180 (1803). Therefore, the complete omission of

the fact that a claim not authorized under significant scientific agreement may still have to be under the First Amendment is derelict of the agency. Indeed, the omission from the Guidance of reference to the *Pearson* Court's disclaimer requirement to protect First Amendment rights is a glaring one that renders the Guidance false and misleading. Its omission is material because regulatees may perceive that FDA's failure to authorize a claim under significant scientific agreement condemns the claim to indefinite suppression when, in fact, the constitutional duty of this agency is to authorize all, at worst, potentially misleading claims with corrective disclaimers. FDA must revise the Guidance to make clear to the regulated class that a claim it deems not backed by "significant scientific agreement" will nevertheless be authorized when a disclaimer can render it nonmisleading.

E. FDA'S GUIDANCE VIOLATES THE NLEA BY FAILING TO DEFINE "SIGNIFICANT SCIENTIFIC AGREEMENT" AS CONGRESS INTENDED

Congress has been severely critical of the way in which FDA has interpreted "significant scientific agreement." See Senate Report No. 103-410. In fact, Congress has documented the existence of an unscientific agency bias against dietary supplements and dietary supplement health claims that it has found wholly inconsistent with the intended meaning of "significant scientific agreement." The following are among Congress' findings on agency bias against claim approval:

> **In fact, the FDA has had a long history of bias against dietary supplements. S.Rep.No. 103-410, at 14 (1994).**
>
> **Mindful of the persistent evidence of FDA bias against dietary supplements . . . S.Rep.No. 103-410, at 30 (1994).**

> **Given the FDA's historical bias against dietary supplements. . . S.Rep.No. 103-410, at 31 (1994).**
>
> **Despite a voluminous scientific record indicating the potential health benefits of dietary supplements, the Food and Drug Administration has pursued a heavy-handed enforcement agenda against dietary supplements for over 30 years. S.Rep.No. 103-410, at 14 (1994).**
>
> **FDA's treatment of health claims on dietary supplements and its implementation of the health claims standard is hindering, rather than fostering, the dissemination of truthful and nonmisleading information about the nutrient/disease rleationship. S.Rep.No. 103-410, at 23 (1994).**
>
> **The committee has heard multiple complaints that the FDA has been overly slow and rigid in considering and approving health claims for dietary supplements. S.Rep.No. 103-410, at 30 (1994).**
>
> **FDA has applied [its health claims review standard] in a way that limits consumer access to important information on diet and health. S.Rep.No. 103-410, at 23 (1994).**
>
> **The FDA has acted to restrict the information that the public may receive about dietary supplements. S.Rep.No. 103-410, at 16 (1994).**
>
> **Despite the fact that the scientific literature increasingly reveals the potential health benefits of dietary supplements, the Food and Drug Administration has pursued a regulatory agenda, which discourages their use by citizens seeking to improve their health through dietary supplementation. S.Rep.No. 103-410, at 14 (1994).**
>
> **In December, 1991, FDA proposed rules implementing the NLEA, but rejected all but one claim for supplements (for calcium/osteoporosis in White and Asian Women). Only one other claim has been approved since that time, the claim for folic acid and neural tube defects, and that claim was only approved after intense public pressure on the FDA. S.Rep.No. 103-410, at 15-16 (1994).**
>
> **The preceding examples show how the FDA has tried to "protect" the public against "unsafe" products for which there is no evidence that the product is unsafe. The FDA has also acted to restrict the information that the public may receive about dietary supplements. Folic acid is a clear example. S.Rep.No. 103-410, at 16 (1994).**

Beholden as it must be to Congress for its statutory authority, FDA has acted in a most peculiar manner. Rather than comply with the dictates of Congress, it has defied them. It

has chosen (against the express congressional command that it not do so) to articulate clearly only one sure way to achieve health claim approval (i.e., establish to FDA's satisfaction that a claim is backed by randomized, controlled clinical trials and direct proof of causation, to wit, establish satisfaction of the drug certainty standard). Congress plainly and unequivocally rejected the drug certainty standard for dietary supplement health claims. It has implored this agency to adopt a definition for significant scientific agreement far less stringent, a definition that FDA does not adopt in the Guidance. In committee Congress has made its expectations clear:

> **The Committee notes that the significant scientific agreement standard is, by design, more flexible than the standard established by law for FDA to review and approve drugs, which requires a demonstration of safety and effectiveness based on "adequate and well-controlled clinical investigations." While the intake of a nutrient on which a health claim is based must be safe, there is no requirement that health claims be derived from clinical trials, and, by its terms, the standard recognizes that scientific agreement on the validity of the claim does not have to be complete. Evidence from a broad range of reliable scientific sources should be considered in determining the adequacy of scientific support.**
>
> **In implementing the significant scientific agreement standard, FDA will be expected to take full advantage of the flexibility of the standard to maximize the availability on food and dietary supplement labels and labeling of disease-related information consumers can prudently use to affect their risk of disease.**
>
> **This includes recognizing that there will nearly always be some remaining scientific uncertainty about the validity of any diet-related health claim; that some individuals consuming or avoiding a nutrient in response to a health claim may benefit, while others may not; and that the benefits for any individual may consist not of absolutely avoiding a disease, but rather of reducing her or his risk of a disease.**
>
> **The end point for evaluation of the adequacy of support for a claim should not be definitive proof that the nutrient has the stated effect for all populations, but that the nutrient will produce the stated effect in the majority of a target population the majority of the time. In addition, the scientific evidence supporting a claim should not be held to the same standard used in evaluating new drug applications.**

> **Under the significant scientific agreement standard, the FDA should authorize claims when a significant segment of scientists having relevant expertise agree, based on relevant scientific evidence, that consumers are reasonably likely to obtain the claimed health benefit. This is consistent with the NLEA's goal of assuring that consumers have access on food and dietary supplement labels to health claims that are scientifically supported, without having to wait until the degree of scientific certainty contemplated by the drug standard has been achieved.**

S.Rep.No. 103-410, at 24.

Thus, FDA's Guidance has violated the intent of Congress by not defining significant scientific agreement as Congress ordered it to in Senate Report No. 103-410. FDA may not interpret significant scientific agreement to have a meaning contrary to that intended by Congress. Indeed, FDA's Guidance is wholly inconsistent with the intent of Congress on interpreting significant scientific agreement under the NLEA. Accordingly, that interpretation is invalid under *Chevron , U.S.A., Inc. v. Natural Resources Defense Council, Inc.,* 467 U.S. 837 (1984) because Congress has spoken to the precise matter in issue and the agency's interpretation is unreasonable in light of congressional intent.

F. JOINT COMMENTERS' RECOMMENDATIONS FOR REVISION TO THE GUIDANCE

The FDA must revise the Guidance if it is to survive judicial review. The Guidance fails to define "significant scientific agreement" as ordered by the *Pearson* Court. The Guidance indicates that a health claim is likely to be approved only if it is backed by randomized, controlled clinical trials and direct proof of causality. That benchmark is far higher than the one intended by Congress for dietary supplement health claims. Moreover, FDA has revealed an unscientific bias in favor of three private associations' opinions and recommendations. Finally, it has omitted from the Guidance the material fact that even if FDA deems a claim not backed by "significant scientific

agreement," it has a constitutional duty nonetheless to authorize even a potentially misleading claim with a corrective disclaimer.

To cure the many defects in the Guidance, FDA should: (1) define "Significant Scientific Agreement" as Congress intended, to wit: "**when a significant segment of scientists having relevant expertise agree, based on relevant scientific evidence, that consumers are reasonably likely to obtain the claimed health benefit;**" (2) should state where on the continuum of scientific evidence between emerging science and consensus "significant scientific agreement" exists consistent with Congressional intent; (3) should state clearly that it will not require the drug certainty standard of proof (i.e., randomized, controlled interventional studies and direct proof of causality) as a condition precedent to dietary supplement health claim approval; (4) should remove reference to the Committee on Nutrition of the American Academy of Pediatrics; the American Heart Association; and the American Cancer Society from the Guidance and make clear that it will not view those organization's opinions or recommendations as in any way more significant than the views of any other private scientific body or private scientific authority; and (5) should include reference to *Pearson*'s constitutional mandate and make clear that if a claim fails to satisfy FDA's "significant scientific agreement" standard it will be authorized nonetheless so long as the addition of a disclaimer can render it nonmisleading.

CONCLUSION

For the foregoing reasons, FDA should immediately discontinue reliance on the Guidance and revise it as recommended herein.

Respectfully submitted,

JULIAN M. WHITAKER, M.D.;
PURE ENCAPSULATIONS, INC.;
XCEL MEDICAL PHARMACY, LTD.;
MYCOLOGY RESEARCH LABORATORIES, LTD.;
DURK PEARSON and SANDY SHAW; and
AMERICAN PREVENTIVE MEDICAL ASSOCIATION,

By _____
Jonathan W. Emord
Claudia A. Lewis-Eng
Eleanor A. Kolton
Counsel for Joint Commenters

Dated: February 22, 2000

Exhibit A

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
Washington DC 20204

OCT 5 1999

Jonathan W. Emord
1050 Seventeenth Street, NW
Suite 600
Washington, DC 20036

Dear Mr. Emord:

This is in response to your letter of September 23, 1999. Your letter made several requests relating to FDA's Federal Register notice of September 8, 1999 (64 Fed. Reg. 48841), which solicited scientific data on the four health claims remanded to the agency in Pearson v. Shalala. Specifically, you requested that FDA (1) extend the time for submitting scientific data on the four claims until 75 days after the agency publishes its guidance on the significant scientific agreement standard; (2) confirm to you in writing and publish a correction notice in the Federal Register clarifying that FDA intends to consider whether the four claims may be authorized with a disclaimer even if the agency determines that they do not meet the significant scientific agreement standard.

With respect to your first request, we agree to extend or reopen the comment period on the September 8, 1999, notice for 75 days after the significant scientific agreement guidance is published. We agree that this is an example of when taking additional time is warranted. Be assured that the agency will give careful consideration to the data that it receives during the second 75 days.

As to your second request, we agree that the court's decision requires FDA to reconsider not only whether each of the four claims meets the significant scientific agreement standard, but also, even if that standard is not met, whether the addition of a disclaimer to the claim could render it non-misleading. If the answer to either question is yes, we will authorize the claim. We do not believe that a Federal Register correction notice is necessary, however. The September 8 Federal Register notice was only intended to solicit scientific data on the four remanded claims, not to describe the procedure and standard the agency will use to evaluate them. The notice stated that FDA was planning to reevaluate the scientific evidence for the claims "as a first step in complying with the court's decision." 64 Fed. Reg. at 48842 (emphasis added). Given the fact that the notice contained no errors and was not intended to explain the court's decision or set forth the agency's plans for implementing the decision, we see no need for a correction notice.

Your concerns about the notice and about statements in FDA's September 17, 1999, letter seem to stem at least in part from a misunderstanding about FDA's use of the word "authorize." By saying that the four claims must be "authorized" by FDA before they may be made in labeling, we meant only that the claims cannot be used unless and until FDA issues a regulation permitting them. We did not mean to imply that we would issue such a regulation only if the claims are found to meet the significant scientific agreement standard.

We hope that the above responds to your concerns.

Sincerely,

Joseph A. Levitt
Director
Center for Food Safety
and Applied Nutrition